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Keywords:	Obesity, SNP, mitochondrial respiratory chain

RESPIRATORY CHAIN POLYMORPHISMS AND OBESITY IN SPANISH POPULATION, A CROSS-SECTIONAL STUDY

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ABSTRACT

OBJECTIVE: To study the association between genes involved in Mitochondrial Respiratory Chain (MRC) pathway with Body Mass Index (BMI) and obesity risk.

DESIGN: This work studies 3 different cross-sectional populations from Spain: HORTEGA and SEGOVIA (center), and PIZARRA (South). These populations represent zones with different characteristics.

SETTING: Forty-eight SNPs from MRC genes were selected and genotyped by SNPlex method. Association studies with BMI and obesity risk were performed for each population. These associations were then verified by carrying out the analysis in the whole studied population (3731 samples).

PARTICIPANTS: a total of 3731 Caucasian individuals: 1502 samples from HORTEGA, 988 from PIZARRA and 1241 from SEGOVIA.

RESULTS: Associations between rs4600063 (SDHC), rs11205591 (NDUFS5), rs10891319 (SDHD) SNPs, BMI and obesity risk were observed (BMI p values 0.04, 0.0011 and 0.0004, respectively. Obesity risk p values 0.0072, 0.039 and 0.0038). In addition, rs11205591 and rs10891319 polymorphisms showed an epistatic interaction for BMI levels and obesity risk.

CONCLUSIONS: Several polymorphisms from genes coding MRC proteins may be involved in BMI variability and could be related to the risk to become obese in the Spanish general population.

KEYWORDS: Obesity, SNP, mitochondrial respiratory chain,

STRENGHTS AND LIMITATIONS OF THIS STUDY:

- This work has been performed in 3 general populations from Spain, with different characteristics.
- Unless the number of individuals in this study is limited, the statistical power is sufficient for the number of analyzed SNPs.
- Results from this work should be confirmed with larger sample sizes.

INTRODUCTION

Obesity is a metabolic disorder consisting of excess body fat accumulation. Obesity prevalence has been increasing during the last decades in Western Societies, becoming one of the most important public health problems. This is due to the fact that obesity is causally related to several chronic diseases such as insulin resistance (IR), cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM).[1]

Susceptibility to obesity is determined by environmental and genetic factors. Although the increment of obesity prevalence is triggered by changes in lifestyle, the risk to develop obesity has an important genetic component, which has been considered in a large number of studies.[2-4] Rare mutations in genes encoding for appetite-regulating proteins, such as leptin (*LEP*) and its receptor (*LEPR*), melanocortin-4 receptor (*MC4R*) and pro-opiomelanocortin (*POMC*), have shown to cause severe early-onset obesity.[5-6] Genome wide association studies (GWAS) have allowed for the identification of common polymorphisms located in, or near to, 97 loci, most of them expressed in the central nervous system.[6-10] However, it is also known that, due to limitations of GWAS, studies around 250 common variants with a similar effect to those described previously remain to be identified.[8]

The study of target genes or genes involved in a particular physiological pathway is a

potential approximation for identification of genetic variants with roles in complex traits. The mitochondrial respiratory chain (MRC) is a biological system involved in carbohydrate and lipid metabolism through oxidative phosphorylation. MRC produces the proton gradient needed for ATP synthesis and is composed by four large complexes (I to IV) that transport electrons from donors (NADH at complex I, FADH2 at complex II) to acceptors. Finally, electrons in complex IV are conducted to the last acceptor, the molecular oxygen.[11] Mitochondrial oxidative capacity dysfunction in liver, muscle or adipose tissue could contribute to the intracellular accumulation of fatty acids observed in obesity.[12-13] Insulin resistance has been associated to lower mitochondria activity at rest.[14] This may be the result of inherited defects in genes coding MRC proteins.

Mutations of MRC genes resulting in loss of function have been described to produce diseases with important disabilities.[15-18] In addition, studies on their impact on energy efficiency and expenditure have shown an association with the risk to develop obesity.[12, 19-21] On the other hand, studies have shown that obesity affects the function of mitochondria, leading to mitochondrial dysfunction.[22] Mitochondrial dynamics play a role in metabolism and obesity: MFN2, a gene coding for a GTPase in the outer mitochondrial membrane is involved in mitochondrial fusion, regulating the operation of the mitochondrial network in skeletal muscle. When MFN2 is repressed, glucose oxidation and mitochondrial membrane potential are impaired.[23] It has been shown that expression of MNF2 is repressed in obese patients compared to lean.[23] Furthermore, polymorphisms in MRC genes, or others related to its regulation, have been related to obesity in different populations.[23-25]

The aim of this study was to find associations between MRC nuclear genes, BMI and obesity in three different Spanish studies in the general population.

MATERIALS AND METHODS

Sample Populations

We have independently analyzed 3 general Spanish populations, which were originally recruited for the study of cardiovascular risk factors and cardiovascular disease development: HORTEGA (1502 subjects collected from 2004 to 2005), PIZARRA (988 individuals, collected from 2009 and 2010) and SEGOVIA (1239 subjects, collected between 2000 and 2003) studies. [26-28] The HORTEGA sample involves subjects from the Valladolid area (North-western Spain). The PIZARRA sample comprises subjects from Pizarra, a town in the Malaga province (Andalusia, Southern Spain). The SEGOVIA sample comprises subjects from the Segovia Province (Center of Spain). These 3 populations were recruited to identify cardiovascular risk factors in general population as a general goal. Individuals were randomly selected and invited to participate. Exclusion criteria were any concomitant disease or condition that prevented them from answering a survey, sample donation or influencing the collection of reliable information. The studies were approved by the specific institutional Research and Ethics Committees, and all patients signed an informed consent to take part in the investigations. The research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki)

Demographic data and anthropometric parameters were collected under standard procedures. Presence of obesity, hypertension (HTN) and Type 2 Diabetes mellitus (T2DM) was registered. BMI was calculated by dividing weight in kg by height in meters squared. Obesity, HTN and T2DM were defined using the World Health Organization (WHO) criteria (http://www.who.int). Briefly, obesity was diagnosed with a BMI >30 kg/m2 and HTN was

defined by systolic and diastolic blood pressure above 140 or 90 mm Hg, respectively. T2DM was defined by fasting plasma glucose ≥ 126mg/dl or 2-hour plasma glucose ≥ 200mg/dl. Previous diagnosis of T2DM or HTN and detection of the disease at the moment of sample collection were registered. Missing data for parameters regarding this work were (expressed as HORTEGA missing data / PIZARRA missing data / SEGOVIA missing data) 58/58/6 for BMI and 1/85/63 for glucose.

Genotyping methods

Genes and SNPs selection:

Forty-eight single nucleotide polymorphisms (SNPs) of chromosomal genes coding for MRC proteins were selected for genotyping. Selection was performed based on the following considerations: functionality (previously described or possible effect), minor allele frequency (MAF) ≥1%, representation of genetic variability of the whole gene (HapMap polymorphisms), and spacing along the gene. Most important variants described in the literature were included. Details of genes and SNPs included are shown as supplemental material (Table 1S).

Genotyping procedure:

Venous blood samples were collected in tubes containing EDTA. DNA was isolated by standard commercial procedures (Chemagic Magnetic Separator from Chemagen, Baesweiler, Germany). DNA was quantified and diluted to a final concentration of 100 ng/ul.

SNPlex (Applied Biosystems, Foster City, California, USA) was used for genotyping, following the manufacturer's guidelines. SNPlex is a genotyping system based on oligonucleotide ligation assay/polymerase chain reaction technology that analyzes 48 SNPs. Those SNPs were chosen as explained above.

Statistical analysis

Statistical analyses were performed using SPSS version 19 and SNPStats software.[29]

Analysis of variance and chi-squared test were used to compare quantitative and categorical variables between groups in order to assess general characteristics of the studied populations. P-values ≤0.05 were considered significant.

When analyzing for associations between SNPs and obesity traits in the 3 populations, 11 out of the 48 SNPs were excluded from the study because they did not fulfill the Hardy-Weimberg equilibrium, had low frequency, or were not detectable by the genotyping procedure. The Bonferroni correction cut off to assess significant associations was calculated for the 37 remaining SNPs. Those polymorphisms associated in at least one out of the three populations and showing the same tendency in the remaining ones, or results with p-values near the significance cut off point in the 3 studies, were also analyzed in the 3729 total subjects from the 3 populations. For these analyses, multivariate logistic regression under co-dominant model was performed and, if applicable, dominant, recessive, or additive models were used. For categorical variables, adjusted odds ratios were assessed with 95% confidence interval. The association between polymorphisms and BMI values was examined using analysis of covariance (ANCOVA). All p-values were two-sided. All results were obtained after adjustment by age and gender, and p-values <0.05 were considered significant.

Patient and Public involvement

Patients and public were not actively involved in this research. They were informed regarding the research goals, protocol development and parameters to be measured before starting the study. If appropriate, they were informed regarding results.

RESULTS

Characteristics of the studied populations

General characteristics of the studied populations are summarized in Table 1. Age, gender and BMI were different between the 3 populations. There were differences in obesity and T2DM percentage: obesity prevalence was 17%, 26% and 33% for Hortega, Segovia and Pizarra studies, respectively, while T2DM prevalence was 7.6%, 10.2% and 19.6%

Table 1: General features of Hotega, Pizarra and Segovia patients considered individually and as a whole population

	Hortega	Pizarra	Segovia	Whole Population
N	1502	988	1241	3731
Age (Years)	54.4 ± 19.3*	46.1 ± 13.9***	51.9 ± 10.8**	51.4 ± 15.9
Height (m)	163.7 ± 10.0*	161 ± 8.8	161.4 ± 9.0**	162.2 ± 9.4
Weight (Kg)	70.8 ± 12.9*	74.1 ± 4.3***	72.1 ± 12.4**	72.1 ± 13.2
BMI (Kg/m2)	26.4 ± 4.2*	28.6 ± 5.3***	27.7 ± 4.3**	27.4 ± 4.6
Gender (M(%)/F(%))	754(50.2) / 748(49.8)*	365(36.9) / (616(62.3)***	562(45.3) / (679(54.7)**	2043(54.8) / (1681(45.1)
Obesity (N(%))	262(17.4)*	331(33.5)***	319(25.7)**	912(24.4)

M: Male. F: Female. Values expressed as mean ± SD except for gender and obesity. P value <0.05was considered significant. *for p<0.05 when comparing Hortega with Pizarra, ** p<0.05 when comparing Hortega and Segovia, *** for p<0.05 when comparing Pizarra and Segovia

Association between MRC genes SNPs and obesity

We performed the analysis looking for associations between SNPs, BMI and obesity risk in the 3 independent populations. Table 2 shows results for those SNPS with differences in at least one of the 3 populations and a similar trend in the remaining ones, or that had p-values near the significance cut off point ($p \leq 0.05$) in the 3 studies: rs1136224 (NDUFS2 gene), rs11205591 (NDUFS5), rs4600063 (SDHC), rs683943 (COX7A2), rs3770989 (NDUFS1) and rs10891319 (SDHD). These 6 SNPs were selected for further analysis in the whole population.

Table 2: BMI and obesity risk of associated SNPs for Hortega, Pizarra and Segovia populations.

		HORTEGA			PIZARRA			SEGOVIA		
	Geno-type	**N**	**BMI (Kg/m2)**	**Obesity OR (95%CI)**	**N**	**BMI (Kg/m2)**	**Obesity OR (95%CI)**	**N**	**Obesity OR (95%CI)**	**Obesity OR (95%CI)**
SDHC	AA	1250	26.46±0.12	1	772	28.70±0.19	1	943	27.71±0.10	1
rs4600063	AG-GG	183	26.16±0.30	0.82 (0.55-1.22)	96	27.86±.04	0.47 (0.28-0.78)	132	27.21±0.30	0.88 (0.57-1.36)
HWE:1	p-value		0.9	0.33		0.071	**0.0025**		0.22	0.57
COX7A2	CC-CG	1410	26.40±0.11	1	865	28.58±0.10	1	1074	27.62±0.10	1
rs683943	GG	4	28.76±2.16	2.65 (0.33-21.1)	4	32.33±0.30	NA (0.00-NA)	2	31.18±4.60	3.17 (0.19-52.05)
HWE:0.6	p-value		0.3	0.36		**0.044**	**0.001**		0.22	0.43
NDUFS1	TT	1308	26.41±0.12	1	799	28.49±0.10	1	1028	27.56±0.10	1
rs3770989	CT-CC	114	26.62±0.44	1.13 (0.72-1.77)	77	29.85±0.60	1.5 (0.91-2.48)	65	27.92±0.60	1.28 (0.73-2.23)
HWE:1	p-value		0.34	0.6		**0.016**	0.12		0.48	0.39
NDUFS2	AA-AG	1379	26.43±0.11	1	838	28.59±0.10	1	1051	27.57±0.10	1
rs1136224	GG	35	26.12±0.79	2.34 (1.11-4.91)	25	29.12±0.70	1.24 (0.52-2.95)	20	28.83±0.90	2.47 (1.00-6.06)
HWE:0.36	p-value		0.65	**0.047**		0.66	0.63		0.27	0.055
NDUFS5	CC-CG	1309	26.47±0.12	1	832	28.63±0.10	1	947	27.6±0.14	1
rs11205591	GG	115	25.78±0.31	0.67 (0.41-1.10)	38	27.66±0.70	0.86 (0.41-1.81)	89	27.21±0.40	0.89 (0.53-1.5)
HWE:0.76	p-value		**0.049**	0.11		0.38	0.69		0.25	0.66
SDHD	AA	691	26.16±0.16	1	401	28.18±0.26	1	571	27.37±0.17	1
rs10891319	AG-GG	726	26.64±0.16	1.16 (0.90-1.49)	478	29.00±0.24	1.22 (0.91-1.64)	492	27.83±0.19	1.27 (0.96-1.68)
HWE:0.22	p-value		0.08	0.25		0.084	0.18		0.077	0.095

BMI: mean ± SD . OR: odds ratio; 95%CI: 95% confidence interval. HWE: Hardy Weinberg Equilibrium. N: Number of individuals. Bold: significant

When analyzing these 6 SNPS in the total population, there were 4 polymorphisms associated to BMI: rs4600063, rs10891319, rs3770989, rs11205591, located in the SDHC, SDHD, NDUFS1 and NDUFS5, genes respectively. Results for these 4 SNPs are shown in Table 3: rs4600063 and rs3770989 showed low association levels with BMI that did not remain

significant after Bonferroni correction (p<0.0013). Rs10891319 and rs11205591 showed significant associations with BMI after Bonferroni correction, with a p-value of 0.0004 for the first one and 0.0011 for the second one. On the other hand, rs4600063 and rs11205591 reduce obesity risk, while rs10891319 increases the risk (p<0.05).

Table 3: SNP Genotyping: distribution of BMI levels and the obesity risk associated between all genotypes in whole population.

Gene/SNP	Genotype	N	BMI (Kg/m^2)	Non Obesity (N(%))	Obesity (N(%))	OR (95% CI)
SDHC	AA	2965	27.44±0.08	2109(86.9)	851(90.4)	1
rs4600063	AG-GG	411	26.89±0.21	318(13.1)	90(9.6)	0.72 (0.56-0.92)
	p-value		**0.04**			**0.0072**
SDHD	AA	1663	27.06±0.11	1243(51.3)	422(45.4)	1
rs10891319	AG-GG	1696	27.60±0.11	1178(48.7)	508(54.6)	1.25 (1.08-1.46)
	p-value		**0.0004**			**0.0038**
NDUFS1	TT	3135	27.32±0.08	2273(92.9)	857(91.2)	1
rs3770989	CT-CC	256	27.92±0.32	173(7.1)	83(8.8)	1.30 (0.99-1.72)
	p-value		**0.026**			0.066
NDUFS5	CC-CG	3088	27.40±0.08	2219(92.3)	863(94)	1
rs11205591*	GG	242	26.60±0.24	184(7.7)	55(6)	0.72 (0.52-0.99)
	p-value		**0.0011**			**0.039**

OR: odds ratio; 95%CI: confidence interval 95%. Values of BMI are expressed as mean ± standard deviation. Results after adjustment for the following variables included in the model: age and gender. N: Number of individuals. *tag-SNP.

Figure 1 shows BMI differences for rs10891319 and rs11205591 polymorphisms in each independent population and the whole one. A similar trend may be observed in both figures 1A

and 1B, although only the rs11205591 SNP reaches significant association at the HORTEGA study ($p \leq 0.05$).

An additive effect for rs11205591 and rs10891319 SNPs was found in the whole sample: patients carrying GG/AA combination presented significant lower BMI (Figure 2A) and lower obesity risk (Figure 2B) than patients carrying CC-CG / AG-GG genotypes (data are expressed as rs11205591 genotype / rs10891319 genotype).

DISCUSSION

The goal of this population-based study was to analyze MRC SNPs and their association with BMI and obesity risk in three different Spanish populations. Those SNPs with the most relevant associations were tested in the pooled sample.

Our group has found a significant protective association for the risk of obesity for rs10891319 AA carriers and for patients with the rs11205591 GG genotype, who also showed reduced BMI. Despite differences in age, sex, BMI and obesity prevalence between the three samples, a similar trend was found considering them individually, while that which corresponded to rs10891319 was the strongest at the whole sample. Furthermore, an epistatic interaction was observed between these 2 SNPs, being 1.4 kg/m2 the maximal BMI difference found between genotypes (CC-GG / AA-GG vs GG / AA carriers, expressed as rs11205591/ rs10891319 genotype carriers). In agreement with these findings, significant differences for obesity risk were also found.

Another SNP, rs3770989, associated with BMI in one of the three populations and in the whole group, is located in the 3'UTR region of the NDUFS1 gene, which codes for NADH dehydrogenase (ubiquinone) Fe-S protein 1. In silico analysis at TargetScanHuman program (http://www.targetscan.org/vert_50/) showed that this SNP is located between two regions of miRNA binding sites, although it does not seem to affect any of them.[30,31] On the other hand, the rs11205591 and rs10891319 SNPs are located in 3' regions of NDUFS5 and SDHD genes respectively. Although these SNPs have an unknown functional effect, they could be in linkage disequilibrium with other truly functional polymorphisms. New studies should be performed to address this question.

The first gene consistently identified as an obesity gene using GWAS was FTO. Several SNPs located on its first intron were associated with BMI in European, East Asian and African populations. [32,33] Following this gene, 75 additional obesity loci have been identified using this methodology and, although FTO is the gene accounting for most of the inter-individual variability on BMI, it only explains a 0.34%.[8,33] There are data supporting that alterations in genes related to mitochondrial function can be involved in obesity, [12,15,20,21,34] but only few studies have analyzed the effect of its variations on BMI or obesity.[19,24] From all genes identified in the GWAS meta-analysis performed by Speliotes and colleages,[8] only the NDUFS3 (NADH dehydrogenase (ubiquinone) Fe-S protein 3) gene was identified to be within 300 Kb from the BMI associated SNPs. As these authors indicated, in spite of the large population analyzed in their study, many genetic variants related to BMI and obesity remain to be identified. The limitations of this meta-analysis come from the different microarrays used, with low coverage in many genomic regions. Therefore, NDUFS5 gene (where rs11205591 is placed) has been analyzed by only one polymorphism in each of the two most used microarrays by Speliotes and in most of the GWAS performed up to now;[8] http://www.Illumina.com;

http://www.affymetrix.com; http://www.ensembl.org). In addition, representation of Mediterranean populations in Speliotes´ and Locke studies is very small, and, to our knowledge, Spanish populations are not included.[8,9] Therefore, many important genetic regions, related to BMI and obesity regulation in these populations may be missing from their work.

The present study supports the hypothesis that genetic variations on MRC genes may be related to obesity susceptibility in the Spanish population. Therefore, these genes could be new therapeutic targets. Genetics of obesity are complex and not well identified yet, and our data may contribute to a better understanding. Further functional studies and association analyses in larger samples and other populations should be carried out to confirm our results.

AUTHOR CONTRIBUTIONS

G.DM-S. obtained genetic data, analyzed and interpreted results and wrote the manuscript**. VGA** obtained genetic data, contributed to its analysis and interpretation and reviewed the manuscript. **JTR, LSBF, MCS, ALS, AC** obtained population data, made critical revisions of the manuscript and contributed to the discussion. **JCME, GRM, RC**, **MTML** contributed to population studies, discussion and reviewed/edited the manuscript. **FJC** and **ABGG** designed the study, wrote the manuscript, contributed to discussion and reviewed/edited manuscript.

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DISCLAIMER

Neither funder played a role in the study design, data analysis or interpretation of the data.

COMPETING INTERESTS

There are not competing interests to declare

DATA SHARING STATEMENT

No additional data are available.

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LEGENDS

Figure 1. BMI differences found for the associated SNPs genotypes for each population analyzed in this study. Values are expressed as BMI difference means, showing 95% confidence interval. **A**) rs10891319 BMI differences between AG-GG and AA genotypes, taking as reference the first one (BMI difference=0 for carriers of AG-GG genotype). **B)** rs11205591 BMI differences for CC-CG and GG genotypes, with the first one as reference (CC-CG genotype BMI difference=0).

Figure 2. Additive effect of rs10891319 and rs11205591 genotypes combination in BMI and obesity risk. **A)** BMI means. * indicates a p-value < 0.005. **B)** Obesity risk ORs . Rs11205591 CC-CG / rs10891319 AA genotypes were taken as reference (OR=1). The obtained p-value was <0.003. Error bars show standard error. Data are expressed as rs11205591 / rs10891319 genotypes

Figure 1

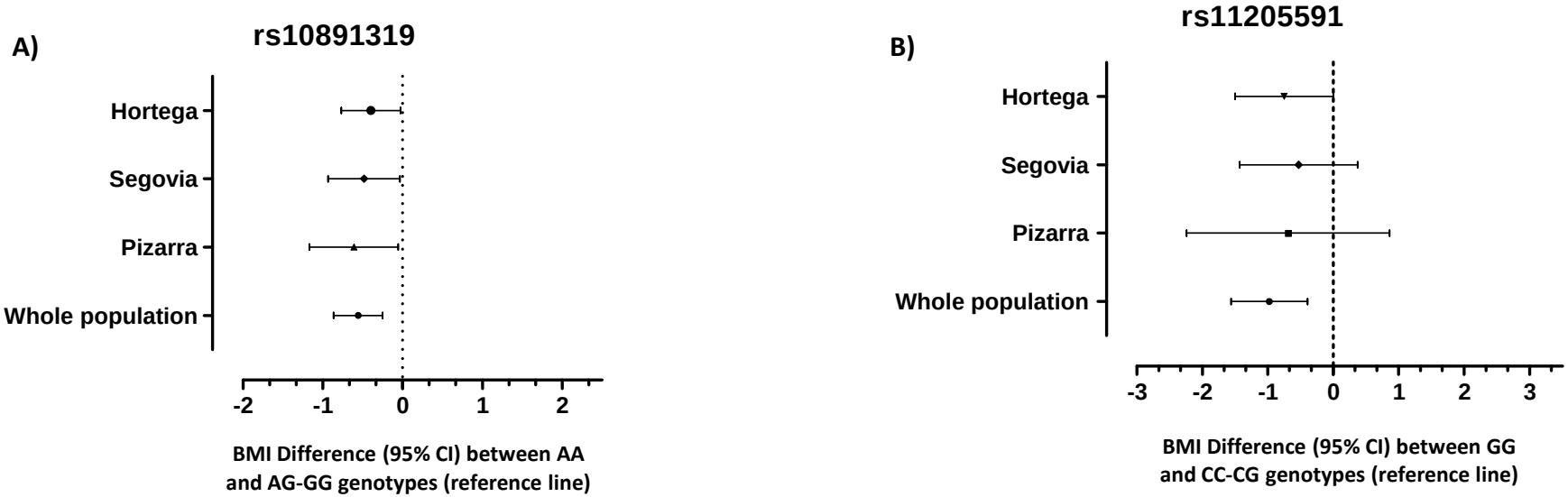
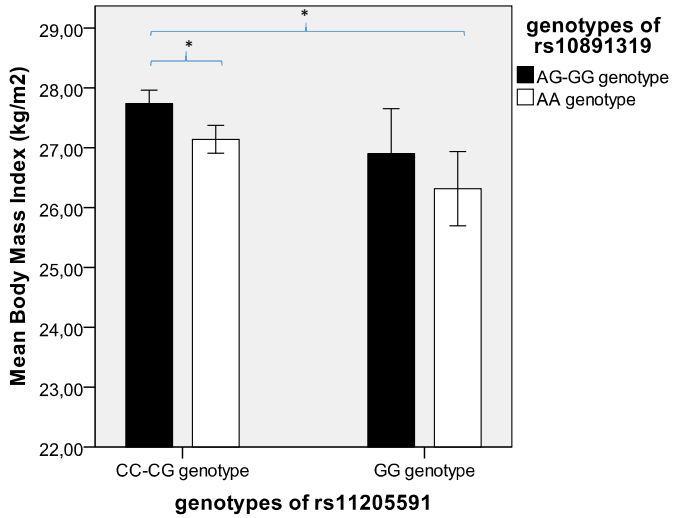

A)
rs10891319
Hortega
Segovia
Pizarra
Whole population
-2
-1
0
1
2
BMI Difference (95% CI) between AA and AG-GG genotypes (reference line)
B)
rs11205591
Hortega
Segovia
Pizarra
Whole population
-3
-2
-1
0
1
2
3
BMI Difference (95% CI) between GG and CC-CG genotypes (reference line)

Figure 2.

A)


Mean Body Mass Index (kg/m2)
29,00
28,00
27,00
26,00
25,00
24,00
23,00
22,00
*
*
genotypes of rs10891319
AG-GG genotype
AA genotype
CC-CG genotype
GG genotype
genotypes of rs11205591

B)

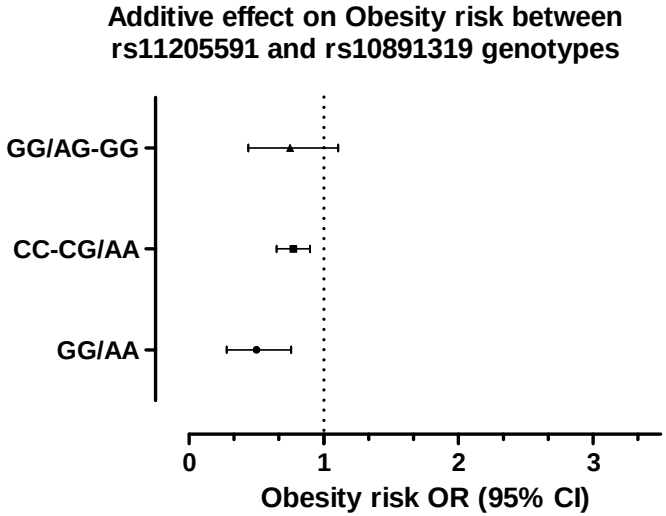

Additive effect on Obesity risk between rs11205591 and rs10891319 genotypes
GG/AG-GG
CC-CG/AA
GG/AA
0
1
2
3
Obesity risk OR (95% CI)

Table 1S: Description of selected SNPs

Gene Name	Chromosome	SNP Name	chromosome position (GRCh38p10)	Most severe consequence	HGVS Name	Reference sequence	MAF	tag SNP
COX5A	15	rs1133322	74920016	DOWNSTREAM	c.*436T>C	NM_004255.3	0.25 (G)	1**
COX5B	2	rs1470625	97649028	DOWNSTREAM	g.97649028C>A	NC_000012.12	0.46 (C)	2**
		rs17022045	97645225	UPSTREAM	c.-862C>G	NM_001862.2	0.13 (G)	1**
COX6A1	12	rs17431357	120442631	UPSTREAM	g.120442631C>T	NC_000012.12	0.04 (T)	
		rs12310837	120442769	UPSTREAM	g.120442769A>G	NC_000012.12	0.15 (G)	
		rs2076022	120437987	UPSTREAM	c.-140C>T	NM_004373.3	0.49 (T)	2**
COX6B1	19	rs4806187	35658859	DOWNSTREAM	c.*212A>G	NM_001863.4	0.33 (A)	1**
COX6C	8	rs1130569	99887565	SYNONYMOUS_CODING	p.Tyr56=	NP_004365.1	0.25 (A)	
		rs4626565	99878314	INTRONIC	c.*16-49A>G	NM_004374.3	0.23 (C)	1**
COX7A1	19	rs753420	36152793	5PRIME_UTR	c.-386A>C	NM_001864.3	0.40 (G)	1**
		rs7255180	36149417	INTRONIC	c.722-161C>T	NM_001003962.2	0.02 (T)	
COX7A2	6	rs436898	75235832	DOWNSTREAM	g.75235832A>C	NC_000006.12	0.16 (A)	
		rs683943	75252092	UPSTREAM	g.75252092C>G	NC_000006.13	0.05 (C)	
		rs9360898	75243989	5PRIME_UTR	c.-159A>C	NM_001865.3	0.21 (G)	3**
COX7B2	4	rs9790574	46732884	DOWNSTREAM	g.46732884C>T	NC_000004.12	0.25 (T)	
COX7C	5	rs16902285	86621753	DOWNSTREAM	g.86621753T>G	NC_000005.10	0.07 (G)	
		rs13161296	86617233	UPSTREAM	c.-823C>T	NM_001867.2	0.01 (T)	1**
		rs2410718	86622655	DOWNSTREAM	g.86622655G>A	NC_000005.10	0.12 (A)	
COX8C	14	rs2089095	93350593	INTRONIC	c.-351+17070C>G	NM_020818.4	0.11 (G)	
NDUFS1	2	rs1053517	206122291	DOWNSTREAM	g.206122291A>G	NC_000002.12	0.49 (G)	1**
		rs3770989	206124027	3PRIME_UTR	c.*158T>C	NM_001199981.1	0.03 (G)	3**
		rs11548670	206147759	SYNONYMOUS_CODING	p.Asp102=	NP_001186910.1	0.07 (G)	2**

Table 1S cont: Description of selected SNPs

Gene Name	Chromosome	SNP Name	chromosome position (GRCh38p10)	Most severe consequence	HGVS Name	Reference sequence	MAF	tag SNP
NDUFS2	1	rs4656994	161210087	INTRONIC	c.703-24G>A	NM_001166159.1	0.26 (A)	3**
		rs1136224	161214307	3PRIME_UTR	c.*366A>G	NM_001166159.1	0.18 (G)	6**
		rs1136207	161213726	SYNONYMOUS_CODING	p.Ala430=	NP_001159631.1	0.16 (T)	
		rs4656993	161206347	INTRONIC	c.203-60A>G	NM_001166159.1	0.30 (A)	
		rs11587213	161215085	UPSTREAM	g.161215085A>G	NC_000001.11	0.15 (G)	5**
NDUFS3	11	rs10742816	47585432	UPSTREAM	g.47585432C>T	NC_000011.10	0.25 (C)	1**
NDUFS4	5	rs31304	53646253	SYNONYMOUS_CODING	p.Gly66=	NP_001304980.1	0.06 (A)	
		rs567	53683267	3PRIME_UTR	c.*46G>A	NM_002495.3	0.38 (A)	3**,5**,6**
		rs535277	53658684	INTRONIC	c.424+60T>G	NM_002495.3	0.29 (G)	
NDUFS5	1	rs1984600	39026255	UPSTREAM	c.-150G>C	NM_004552.2	0.46 (G)	1**
		rs11205591	39035577	DOWNSTREAM	g.39035577C>G	NC_000001.11	0.24 (G)	2**
NDUFS6	5	rs11953620	1818516	DOWNSTREAM	g.1818516C>T	NC_000005.10	0.42 (T)	
		rs4975851	1817222	DOWNSTREAM	g.1817222C>T	NC_000005.10	0.38 (C)	
NDUFS7	19	rs11666067	1389518	non coding transcript exon variant	c.228+580C>A	NM_024407.4	0.46 (A)	
SDHB	1	rs1022580	17033181	INTRONIC	c.201-36G>T	NM_003000.2	0.04 (C)	1**
		rs2647169	17044728	INTRONIC	c.200+33G>A	NM_003000.2	0.11 (T)	3**
		rs11203280	17016288	UPSTREAM	g.17016288G>T	NC_000001.11	0.50 (G)	
		rs9435739	17016429	UPSTREAM	g.17016429G>A	NC_000001.11	0.48 (A)	

Table 1S cont2: Description of selected SNPs

Gene Name	Chromosome	SNP Name	chromosome position (GRCh38p10)	Most severe consequence	HGVS Name	Reference sequence	MAF	tag SNP
SDHC	1	rs4600063	161363401	3PRIME_UTR	g.161363401A>G	NC_000001.11	0.17 (G)	
		rs13374037	161365491	3PRIME_UTR	g.161365491T>A	NC_000001.11	0.17 (A)	3**
SDHD	11	rs10891319	112096881	INTRONIC	g.112096881A>G	NC_000011.10	0.35 (G)	
UQCRB	8	rs10504961	96227901	3PRIME_UTR	g.96227901C>T	NC_000008.11	0.37 (T)	
UQCRQ	5	rs803224	132870812	UPSTREAM	g.132870812G>A	NC_000005.10	0.18 (G)	
		rs17624157	132868339	UPSTREAM	g.132868339G>A	NC_000005.10	0.05 (A)	
GDF9	5	rs39830	132865726	UPSTREAM	g.132865726G>A	NC_000005.10		
		rs30177	132866719	5PRIME_UTR	g.132866719C>G	NC_000005.10	0.36 (C)	3**,4**

SNP Name: dbSNP 149, Ensemble release 89. MAF: Minor allele frequency. HGVS name: nomclature following Human Genome Variation Society guidelines

Table 1. STREGA reporting recommendations, extended from STROBE Statement

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Title and Abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract.		1
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found.		2
Introduction				
Background rationale	2	Explain the scientific background and rationale for the investigation being reported.		3-4
Objectives	3	State specific objectives, including any pre-specified hypotheses.	***State if the study is the first report of a genetic association, a replication effort, or both.***	4
Methods				
Study design	4	Present key elements of study design early in the paper.		5-6

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Setting	5	Describe the setting, locations and relevant dates, including periods of recruitment, exposure, follow-up, and data collection.		5
Participants	6	(a) **Cohort study –** Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up. **Case-control study –** Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls. **Cross-sectional study –** Give the eligibility criteria, and the sources and methods of selection of participants.	***Give information on the criteria and methods for selection of subsets of participants from a larger study, when relevant***.	5
		(b) **Cohort study –** For matched studies, give matching criteria and number of exposed and unexposed. **Case-control study –** For matched studies, give matching criteria and the number of controls per case.		NA
Variables	7	*(a)* Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable.	***(b) Clearly define genetic exposures (genetic variants) using a widely-used nomenclature system. Identify variables likely to be associated with population***	6

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			stratification (confounding by ethnic origin).	
Data sources measurement	8*	*(a)* For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group.	***(b) Describe laboratory methods, including source and storage of DNA, genotyping methods and platforms (including the allele calling algorithm used, and its version), error rates and call rates. State the laboratory/centre where genotyping was done. Describe comparability of laboratory methods if there is more than one group. Specify whether genotypes were assigned using all of the data from the study simultaneously or in smaller batches.***	5-6
Bias	9	*(a)* Describe any efforts to address potential sources of bias.	***(b) For quantitative outcome variables, specify if any investigation of potential bias resulting***	

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			from pharmacotherapy was undertaken. If relevant, describe the nature and magnitude of the potential bias, and explain what approach was used to deal with this.	NA
Study size	10	Explain how the study size was arrived at.		5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why.	***If applicable, describe how effects of treatment were dealt with.***	5-6
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding.	***State software version used and options (or settings) chosen.***	7
		(b) Describe any methods used to examine subgroups and interactions.		7
		(c) Explain how missing data were addressed.		7

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(d) **Cohort study –** If applicable, explain how loss to follow-up was addressed. **Case-control study –** If applicable, explain how matching of cases and controls was addressed. **Cross-sectional study –** If applicable, describe analytical methods taking account of sampling strategy.		NA
		(e) Describe any sensitivity analyses.		
			(f) State whether Hardy-Weinberg equilibrium was considered and, if so, how.	7
			(g) Describe any methods used for inferring genotypes or haplotypes.	7
			(h) Describe any methods used to assess or address population stratification.	
			(i) Describe any methods used to address multiple	

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			comparisons or to control risk of false positive findings.	7
			(j) Describe any methods used to address and correct for relatedness among subjects	
Results				
Participants	13*	(a) Report the numbers of individuals at each stage of the study – e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed.	***Report numbers of individuals in whom genotyping was attempted and numbers of individuals in whom genotyping was successful.***	5-6
		(b) Give reasons for non-participation at each stage.		5
		(c) Consider use of a flow diagram.		
Descriptive data	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders.	***Consider giving information by genotype.***	5

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Indicate the number of participants with missing data for each variable of interest.		6
		(c) **Cohort study –** Summarize follow-up time, e.g. average and total amount.		NA
Outcome data	15 *	**Cohort study-**Report numbers of outcome events or summary measures over time.	***Report outcomes (phenotypes) for each genotype category over time***	
		Case-control study – Report numbers in each exposure category, or summary measures of exposure.	***Report numbers in each genotype category***	
		Cross-sectional study – Report numbers of outcome events or summary measures.	***Report outcomes (phenotypes) for each genotype category***	10-11
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence intervals). Make clear which confounders were adjusted for and why they were included.		7

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Report category boundaries when continuous variables were categorized.		6
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period.		NA
			(d) Report results of any adjustments for multiple comparisons.	10-11
Other analyses	17	(a) Report other analyses done – e.g., analyses of subgroups and interactions, and sensitivity analyses.		
			(b) If numerous genetic exposures (genetic variants) were examined, summarize results from all analyses undertaken.	10-11
			(c) If detailed results are available elsewhere, state how they can be accessed.	NA
Discussion				
Key results	18	Summarize key results with reference to study objectives.		14

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.		
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.		
Generalizability	21	Discuss the generalizability (external validity) of the study results.		
Other Information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based.		

STREGA = STrengthening the REporting of Genetic Association studies; STROBE = STtrengthening the Reporting of Observational Studies in Epidemiology.

* Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

BMJ Open

RESPIRATORY CHAIN POLYMORPHISMS AND OBESITY IN THE SPANISH POPULATION, A CROSS-SECTIONAL STUDY

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Primary Subject Heading:	Genetics and genomics
Secondary Subject Heading:	Diabetes and endocrinology
Keywords:	Obesity, SNP, mitochondrial respiratory chain

RESPIRATORY CHAIN POLYMORPHISMS AND OBESITY IN THE SPANISH POPULATION, A CROSS-SECTIONAL STUDY

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Word count: excluding title page, abstract, references figures and tables: 2433

ABSTRACT

OBJECTIVE: To study the association of genes involved in the Mitochondrial Respiratory Chain (MRC) pathway with Body Mass Index (BMI) and obesity risk.

DESIGN: This work studies three cross-sectional populations from Spain, representing three provinces: HORTEGA (Valladolid, Northwest/Center), SEGOVIA (Segovia, Northwest/center), and PIZARRA (Malaga,South).

SETTING: Forty-eight SNPs from MRC genes were selected and genotyped by SNPlex method. Association studies with BMI and obesity risk were performed for each population. These associations were then verified by analysis of the studied population as a whole (3731 samples).

PARTICIPANTS: a total of 3731 Caucasian individuals: 1502 samples from HORTEGA, 988 from PIZARRA and 1241 from SEGOVIA.

RESULTS: rs4600063 (*SDHC*), rs11205591 (*NDUFS5*) and rs10891319 (*SDHD*) SNPs were associated with BMI and obesity risk (p values for BMI were 0.04, 0.0011 and 0.0004, respectively, and for obesity risk, 0.0072, 0.039 and 0.0038). However, associations between rs4600063 and BMI, and between these 3 SNPs and obesity risk are not significant if Bonferroni correction is considered. In addition, rs11205591 and rs10891319 polymorphisms showed an additive interaction with BMI and obesity risk.

CONCLUSIONS: Several polymorphisms from genes coding MRC proteins may be involved in BMI variability and could be related to the risk to become obese in the Spanish general population.

KEYWORDS: Obesity, SNP, mitochondrial respiratory chain,

STRENGHTS AND LIMITATIONS OF THIS STUDY:

- This research was conducted in three open populations from different provinces of Spain.
- MRC SNP analysis by SNPlex genotyping method overcomes some of the limitations of GWAS.
- One of the limitations of this study is the reduced size of the populations used
- Despite the limited number of individuals in this study, the statistical power is sufficient for the number of analyzed SNPs.
- Results from this study are promising and should be validated by larger sample sizes

INTRODUCTION

Obesity is a metabolic disorder consisting of excess body fat accumulation. Obesity prevalence has been increasing during the last decades in Western Societies, becoming one of the most important public health problems due to its causally relationship with several chronic diseases such as insulin resistance (IR), cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM).[1]

Susceptibility to obesity is determined by environmental and genetic factors. Although rising obesity prevalence is triggered by lifestyle changes, the risk of developing obesity has an important genetic component, which has been examined in numerous studies.[2-4] Rare mutations in genes encoding for appetite-regulating proteins, such as leptin (*LEP*) and its receptor (*LEPR*), melanocortin-4 receptor (*MC4R*) and pro-opiomelanocortin (*POMC*), have shown to cause severe early-onset obesity.[5-6] Genome wide association studies (GWAS) have identified common polymorphisms located in, or near to, 97 loci, mostly expressed in the central nervous system.[6-

10] However, it is also suggested that, due to limitations of GWAS, there are around 250 common variants with a similar effect to those previously described that remain to be identified.[8]

The study of target genes or genes involved in a particular physiological pathway is a potential approach to identify genetic variants with roles in complex traits. The mitochondrial respiratory chain (MRC) is a biological system involved in carbohydrate and lipid metabolism through oxidative phosphorylation. MRC produces the proton gradient needed for ATP synthesis and is composed of four large complexes (I to IV) that transport electrons from donors (NADH at complex I, FADH2 at complex II) to acceptors. Finally, electrons in complex IV are conducted to the last acceptor, the molecular oxygen. [11] Mitochondrial oxidative capacity dysfunction in liver, muscle or adipose tissue could contribute to the intracellular accumulation of fatty acids observed in obesity.[12-13] Insulin resistance has been associated with lower mitochondria activity at rest.[14] This may be the result of inherited defects in genes coding MRC proteins.

Mutations of MRC genes resulting in loss of function have been reported to produce major disability-causing diseases.[15-18] In addition, studies on their impact on energy efficiency and expenditure have shown an association with the risk of developing obesity.[12, 19-21] Studies have also shown that obesity affects the function of mitochondria, leading to mitochondrial dysfunction.[22] Mitochondrial dynamics play a role in metabolism and obesity: *MFN2*, a gene coding for a GTPase in the outer mitochondrial membrane is involved in mitochondrial fusion, regulating the operation of the mitochondrial network in skeletal muscle. When *MFN2* is repressed, glucose oxidation and mitochondrial membrane potential are impaired.[23] It has been shown that expression of *MNF2* is repressed in obese patients compared to lean.[23] Furthermore, polymorphisms in MRC genes, or others related to its regulation, have been related to obesity in different populations.[23-25]

The aim of this study was to find associations between MRC nuclear genes, BMI and obesity in three different Spanish studies in the general population.

MATERIALS AND METHODS

Sample Populations

We independently analyzed three general Spanish populations originally recruited for the study of cardiovascular risk factors and cardiovascular disease development: HORTEGA (1502 subjects collected from 2004 to 2005), PIZARRA (988 individuals, collected from 2009 and 2010) and SEGOVIA (1239 subjects, collected between 2000 and 2003). [26-28] The HORTEGA sample involves subjects from the Valladolid area (North-western Spain). The PIZARRA sample comprises subjects from Pizarra, a town in the Malaga province (Andalusia, Southern Spain). The SEGOVIA sample studied subjects from the Segovia Province (Center of Spain). These three populations were recruited to identify cardiovascular risk factors in the general population as an overall goal. Individuals were randomly selected and invited to participate. Exclusion criteria were any concomitant disease or condition that prevented them from answering a survey, or donating a sample or that would influence the collection of reliable information. The study was approved by the Research and Ethics Committee from the Valencia University Clinical Hospital and INCLIVA (reference number 2010/013). All patients provided informed written consent to take part in the investigations. The research was carried out according to the Ethical Principles of the World Medical Association (Declaration of Helsinki)

Demographic data and anthropometric parameters were collected following standard

procedures. Presence of obesity, hypertension (HTN) and Type 2 Diabetes mellitus (T2DM) was recorded. BMI was calculated by dividing weight in kilograms by height in meters squared. Obesity, HTN and T2DM were defined using the World Health Organization (WHO) criteria (http://www.who.int). Briefly, obesity was diagnosed with a BMI >30 kg/m2, overweight as BMI between 25.0 and 29.9 kg/m2, and normal weight as BMI≤24.9 kg/m2. HTN was defined by systolic and diastolic blood pressure above 140 or 90 mm Hg, respectively. T2DM was defined by fasting plasma glucose ≥ 126mg/dl or 2-hour plasma glucose ≥ 200mg/dl. Previous diagnosis of T2DM or HTN and detection of the disease at the moment of sample collection were recorded. Missing data for parameters regarding this work (expressed as HORTEGA missing data / PIZARRA missing data / SEGOVIA missing data) were 58/58/6 for BMI and 1/85/63 for glucose.

We have calculated the statistical power for our three samples independently (minor allele frequency (MAF) >0.10, genotype relative risk (1.5)), number of obese and non-obese and prevalence of obesity in each of the populations. Furthermore, the statistical power was over 85% for this conditions in all populations and it increases for increased allele frequency (http://csg.sph.umich.edu/abecasis/cats/gas_power_calculator/index.html)·

Genotyping methods

Gene and SNP selection:

Forty-eight single nucleotide polymorphisms (SNPs) of chromosomal genes coding for MRC proteins were selected for genotyping. Selection was performed based on the following considerations: functionality (previously described or possible effect), MAF ≥1%, representation of genetic variability of the whole gene (HapMap polymorphisms), and spacing along the gene. Most important variants described in the literature were included. Details of genes and SNPs

included are shown as supplemental material (Table 1S).

Genotyping procedure:

Venous blood samples were collected in tubes containing EDTA. DNA was isolated by standard commercial procedures (Chemagic Magnetic Separator from Chemagen, Baesweiler, Germany). DNA was quantified and diluted to a final concentration of 100 ng/ul.

SNPlex (Applied Biosystems, Foster City, California, USA) was used for genotyping, following the manufacturer's guidelines. SNPlex is a genotyping system based on oligonucleotide ligation assay/polymerase chain reaction technology that analyzes 48 SNPs. Those SNPs were chosen as explained above.

Statistical analysis

Statistical analyses were performed using SPSS version 19 and SNPStats software. [29]

Chi-squared test and variance analysis were used to compare quantitative and categorical variables between groups in order to assess general characteristics of the studied populations. P-values ≤ 0.05 were considered significant.

When analyzing for associations between SNPs and obesity traits in the three populations, 11 out of the 48 SNPs were excluded from the study because they did not fulfill the Hardy-Weimberg equilibrium, had low frequency, or were not detectable by the genotyping procedure. Hardy-Weimberg test indicated no loss of heterozigosity in the analyzed populations for these SNPs. The Bonferroni correction cut off to assess significant associations was calculated for the 37 remaining SNPs. Those polymorphisms associated in at least one of the three populations and showing the same tendency in the remaining ones, or results with p-values near the nominal cut off point in the three studies ($p<0.05$), were also analyzed in the 3729 total combined subjects from

the three populations. For these analyses, multivariate logistic regression under co-dominant model was performed and, if applicable, dominant, recessive, or additive models were used. For categorical variables, adjusted odds ratios were assessed with 95% confidence interval. The association between polymorphisms and BMI values was examined using analysis of covariance (ANCOVA). All p-values were two-sided. All results were obtained after adjustment for age and gender, and p-values <0.05 were considered significant.

Patient and Public involvement

Patients and public were not actively involved in this research. They were informed regarding the research goals, protocol development and parameters to be measured before starting the study. If appropriate, they were informed regarding results.

RESULTS

Characteristics of the studied populations

General characteristics of the studied populations are summarized in Table 1. Age, gender and BMI varied between the three populations. There were differences in obesity and T2DM percentage: obesity prevalence was 17%, 26% and 33% for Hortega, Segovia and Pizarra studies, respectively, while T2DM prevalence was 7.6%, 10.2% and 19.6%

Table 1: General features of Hortega, Pizarra and Segovia patients considered individually and as a whole population

	Hortega	Pizarra	Segovia	Whole Population
N	1502	988	1241	3731
Age (Years)	54.4 ± 19.3*	46.1 ± 13.9***	51.9 ± 10.8**	51.4 ± 15.9
Height (m)	163.7 ± 10.0*	161 ± 8.8	161.4 ± 9.0**	162.2 ± 9.4
Weight (Kg)	70.8 ± 12.9*	74.1 ± 4.3***	72.1 ± 12.4**	72.1 ± 13.2
BMI (Kg/m2)	26.4 ± 4.2*	28.6 ± 5.3***	27.7 ± 4.3**	27.4 ± 4.6
Gender M(%)/F(%)	754(50.2) / 748(49.8)*	365(36.9) / (616(62.3)***	562(45.3) / (679(54.7)**	2043(54.8) / (1681(45.1)
Obesity (N(%))	262(17.4)*	331(33.5)***	319(25.7)**	912(24.4)

M: Male. F: Female. Values expressed as mean ± SD except for gender and obesity. p value <0.05was considered significant. *for p<0.05 when comparing Hortega with Pizarra, ** p<0.05 when comparing Hortega and Segovia, *** for p<0.05 when comparing Pizarra and Segovia

Association between MRC genes SNPs and obesity

We performed the analysis looking for associations between SNPs, BMI and obesity risk in the three independent populations. Table 2 shows results for SNPS with differences in at least one of the three populations and a similar trend in the remaining ones, or that had p-values near the nominal cut-off point ($p \leq 0.05$) in the three studies: rs1136224 (*NDUFS2* gene), rs11205591 (*NDUFS5*), rs4600063 (*SDHC*), rs3770989 (*NDUFS1*) and rs10891319 (*SDHD*). These five SNPs were selected for further analysis in the whole population.

Table 2: BMI and obesity risk of associated SNPs for Hortega, Pizarra and Segovia populations.

		HORTEGA			PIZARRA			SEGOVIA		
	Geno-type	**N**	**BMI (Kg/m2)**	**Obesity OR (95%CI)**	**N**	**BMI (Kg/m2)**	**Obesity OR (95%CI)**	**N**	**BMI (Kg/m2)**	**Obesity OR (95%CI)**
SDHC	AA	1250	26.46±0.12	1	772	28.70±0.19	1	943	27.71±0.10	1
rs4600063	AG-GG	183	26.16±0.30	0.82 (0.55-1.22)	96	27.86±.04	0.47 (0.28-0.78)	132	27.21±0.30	0.88 (0.57-1.36)
HWE:1	p-value		0.9	0.33		0.071	**0.0025**		0.22	0.57
NDUFS1	TT	1308	26.41±0.12	1	799	28.49±0.10	1	1028	27.56±0.10	1
rs3770989	CT-CC	114	26.62±0.44	1.13 (0.72-1.77)	77	29.85±0.60	1.5 (0.91-2.48)	65	27.92±0.60	1.28 (0.73-2.23)
HWE:1	p-value		0.34	0.6		**0.016**	0.12		0.48	0.39
NDUFS2	AA	1045	26.37±0.13	1	640	28.61±0.21	1	830	27.55±0.15	1
rs1136224	AG-GG	369	26.59±0.23	1.43 (1.09-1.89)	223	28.59±0.34	1.10 (0.79-1.54)	241	27.77±0.26	1.07 (0.76-1.48)
HWE:0.36	p-value		0.25	**0.011**		0.71	0.58		0.42	0.71
NDUFS5	CC-CG	1309	26.47±0.12	1	832	28.63±0.10	1	947	27.6±0.14	1
rs11205591	GG	115	25.78±0.31	0.67 (0.41-1.10)	38	27.66±0.70	0.86 (0.41-1.81)	89	27.21±0.40	0.89 (0.53-1.5)
HWE:0.76	p-value		**0.049**	0.11		0.38	0.69		0.25	0.66
SDHD	AA	691	26.16±0.16	1	401	28.18±0.26	1	571	27.37±0.17	1
rs10891319	AG-GG	726	26.64±0.16	1.16 (0.90-1.49)	478	29.00±0.24	1.22 (0.91-1.64)	492	27.83±0.19	1.27 (0.96-1.68)
HWE:0.22	p-value		0.08	0.25		0.084	0.18		0.077	0.095

BMI: mean ± SD. OR: odds ratio; 95%CI: 95% confidence interval. HWE: Hardy Weinberg Equilibrium. N: Number of individuals. Bold: significant

Analyzing these five SNPS in the total population, four polymorphisms were associated with BMI: rs4600063, rs10891319, rs3770989, rs11205591, located in the *SDHC, SDHD, NDUFS1* and *NDUFS5,* genes respectively. Results for these four SNPs are shown in Table 3: rs4600063 and rs3770989 showed low association with BMI, which lost significance after Bonferroni correction ($p<0.0013$). Rs10891319 and rs11205591 showed significant association with BMI after Bonferroni correction, with a p-value of 0.0004 for the first one and 0.0011 for the

second one. On the other hand, rs4600063 (genotypes AG and GG) and rs11205591 (GG genotype) reduced obesity risk, while rs10891319 (AG and GG genotypes) and rs1136224 (AG and GG genotypes) increased the risk ($p<0.05$), but they did not reach the Bonferroni cut-off point.

Table 3: SNP Genotyping: distribution of BMI levels and the associated obesity risk for all genotypes in whole population.

Gene/SNP	Genotype	N	BMI (Kg/m²)	Non Obesity (N(%))	Obesity (N(%))	OR (95% CI)
SDHC	AA	2965	27.44±0.08	2109(86.9)	851(90.4)	1
rs4600063	AG-GG	411	26.89±0.21	318(13.1)	90(9.6)	0.72 (0.56-0.92)
	p-value		**0.04**			**0.0072**
SDHD	AA	1663	27.06±0.11	1243(51.3)	422(45.4)	1
rs10891319	AG-GG	1696	27.60±0.11	1178(48.7)	508(54.6)	1.25 (1.08-1.46)
	p-value		**0.0004**			**0.0038**
NDUFS1	TT	3135	27.32±0.08	2273(92.9)	857(91.2)	1
rs3770989	CT-CC	256	27.92±0.32	173(7.1)	83(8.8)	1.30 (0.99-1.72)
	p-value		**0.026**			0.066
NDUFS2	AA	2515	27.33±0.09	1833(76.1)	680(73)	1
rs1136224	AG-GG	833	27.46±0.16	575(23.9)	251(27)	1.20(1.01-1.43)
	p-value		0.31			**0.041**
NDUFS5	CC-CG	**3088**	27.40±0.08	2219(92.3)	863(94)	1
rs11205591*	GG	**242**	26.60±0.24	184(7.7)	55(6)	0.72 (0.52-0.99)
	p-value		**0.0011**			**0.039**

OR: odds ratio; 95%CI: 95% confidence interval. BMI values are expressed as mean ± standard deviation. Results after adjustment for the following variables included in the model: age and gender. N: Number of individuals. *tag-SNP.

Figure 1 shows BMI differences for rs10891319 and rs11205591 polymorphisms in each independent population and as a whole. A similar trend can be observed in both figures 1A and 1B, although only the rs11205591 SNP reaches significance in the HORTEGA study ($p \leq 0.05$).

We studied additive effects for all these SNPs and an additive effect for rs11205591 and rs10891319 SNPs was found in the whole sample: patients carrying GG/AA combination presented significant lower BMI (Figure 2A) and lower obesity risk (Figure 2B) than patients carrying CC-CG / AG-GG genotypes (data are expressed as rs11205591 genotype / rs10891319 genotype).

On the other hand, some of the SNPs were within the gene. We calculated possible haplotypes, with negative results.

DISCUSSION

The goal of this population-based study was to analyze MRC SNPs and their association with BMI and obesity risk in three Spanish populations. SNPs with the most relevant associations were tested in the pooled sample.

We found a significant protective association with the risk of obesity for rs10891319 AA, and for patients with the rs11205591 GG genotype, who also showed reduced BMI. Despite differences in age, sex, BMI, and obesity prevalence between the three samples, a similar trend was found considering them individually, while rs10891319 showed the strongest in the whole sample. Furthermore, an additive interaction was observed between these two SNPs, with a maximal BMI difference of 1.4 kg/m2 found between genotypes (CC-GG / AA-GG vs GG / AA

carriers, expressed as rs11205591/ rs10891319 genotype carriers). In agreement with these findings, significant differences in obesity risk were also found.

Another SNP associated with BMI in one of the three populations and in the whole group, rs3770989, is located in the 3'UTR region of the *NDUFS1* gene, which codes for NADH dehydrogenase (ubiquinone) Fe-S protein 1. In silico analysis in TargetScanHuman (http://www.targetscan.org/vert_50/) showed that this SNP is located between two regions of miRNA binding sites, although it does not seem to affect either of them.[30,31] On the other hand, the rs11205591 and rs10891319 SNPs are located in 3' regions of *NDUFS5* and *SDHD* genes respectively. Although these SNPs have an unknown functional effect, they could be in linkage disequilibrium with other truly functional polymorphisms. New studies are needed to address this question.

The first gene consistently identified as an obesity gene using GWAS was FTO. Several SNPs located on its first intron were associated with BMI in European, East Asian and African populations. [32,33] Following this gene, 75 additional obesity loci have been identified using this methodology and, although FTO is the gene accounting for most of the inter-individual variability on BMI, it only explains a 0.34%.[8,33] There are data supporting that alterations in genes related to mitochondrial function can be involved in obesity, [12,15,20,21,34] but few studies have analyzed the effect of its variations on BMI or obesity.[19,24] From all genes identified in the GWAS meta-analysis performed by Speliotes and colleagues,[8] only the *NDUFS3* (NADH dehydrogenase (ubiquinone) Fe-S protein 3) gene was identified to be within 300 Kb from the BMI associated SNPs. As these authors indicated, in spite of the large population analyzed in their study, many genetic variants related to BMI and obesity remain to be identified. The limitations of this meta-analysis come from the different microarrays used, with low coverage in many genomic regions. Therefore, the *NDUFS5* gene (where rs11205591 is located) has been analyzed

by only one polymorphism in each of the two most used microarrays by Speliotes and in most of the GWAS performed to date;[8] (http://www.Illumina.com; http://www.affymetrix.com; http://www.ensembl.org). In addition, representation of Mediterranean populations in Speliotes and Locke´s studies is very small, and, to our knowledge, Spanish populations are not included.[8,9] Therefore, many important genetic regions, related to BMI and obesity regulation in these populations may be missing from their work.

The present study supports the hypothesis that genetic variations in MRC genes may be related to obesity susceptibility in the Spanish population. Therefore, these genes could be new therapeutic targets. Genetics of obesity are complex and not yet well identified, and our data may contribute to a better understanding. One limitation of this study is the reduced size of the analyzed populations. However, the statistical power is sufficient for the number of analyzed SNPs. On the other hand, we have not studied all the possible SNPs present in these MRC genes. Further functional studies and association analyses in larger samples and other populations should be carried out to confirm our results.

AUTHOR CONTRIBUTIONS

G.DM-S. obtained genetic data, analyzed and interpreted results and wrote the manuscript**. VGA** obtained genetic data, contributed to analysis and interpretation and reviewed the manuscript. **JTR, LSBF, MCS, ALS, and AC** obtained population data, made critical revisions of the manuscript and contributed to the discussion. **JCME, GRM, RC**, and **MTML** contributed to population studies and discussion, and reviewed and edited the manuscript. **FJC** and **ABGG** designed the study, wrote the manuscript, contributed to discussion and reviewed and edited the manuscript.

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DISCLAIMER

Neither funder played a role in the study design, data analysis or interpretation of the data.

COMPETING INTERESTS

There are no competing interests to declare

DATA SHARING STATEMENT

No additional data are available.

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LEGENDS

Figure 1. BMI differences found for the associated SNPs genotypes for each population analyzed in this study. Values are expressed as BMI difference means, showing 95% confidence interval. **A**) rs10891319 BMI differences between AG-GG and AA genotypes, taking the first one (BMI difference=0 for carriers of AG-GG genotype) as reference. **B)** rs11205591 BMI differences for CC-CG and GG genotypes, with the first one as a reference (CC-CG genotype BMI difference=0).

Figure 2. Additive effect of rs10891319 and rs11205591 genotype combination in BMI and obesity risk. **A)** BMI means. * indicates a p-value < 0.005. **B)** Obesity risk ORs . Rs11205591 CC-CG / rs10891319 AA genotypes were taken as a reference (OR=1). The obtained p-value was <0.003. Error bars show standard error. Data are expressed as rs11205591 / rs10891319 genotypes

Figure 1

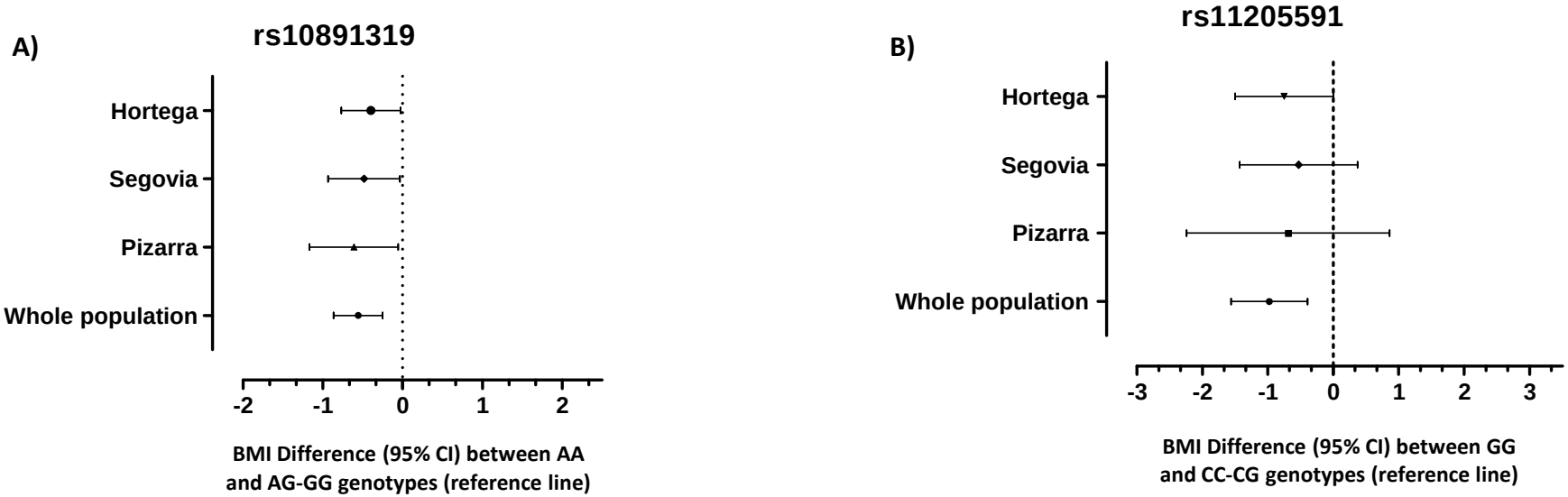
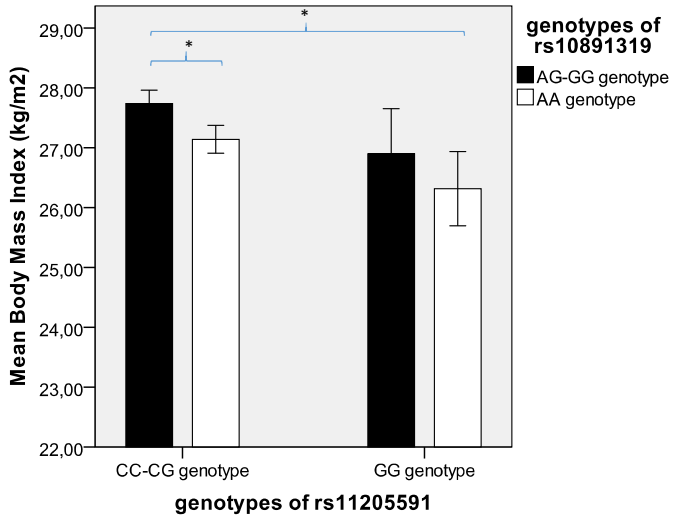

A)
rs10891319
Hortega
Segovia
Pizarra
Whole population
-2
-1
0
1
2
BMI Difference (95% CI) between AA and AG-GG genotypes (reference line)
B)
rs11205591
Hortega
Segovia
Pizarra
Whole population
-3
-2
-1
0
1
2
3
BMI Difference (95% CI) between GG and CC-CG genotypes (reference line)

Figure 2.

A)


Mean Body Mass Index (kg/m2)
29,00
28,00
27,00
26,00
25,00
24,00
23,00
22,00
*
*
genotypes of rs10891319
AG-GG genotype
AA genotype
CC-CG genotype
GG genotype
genotypes of rs11205591

B)

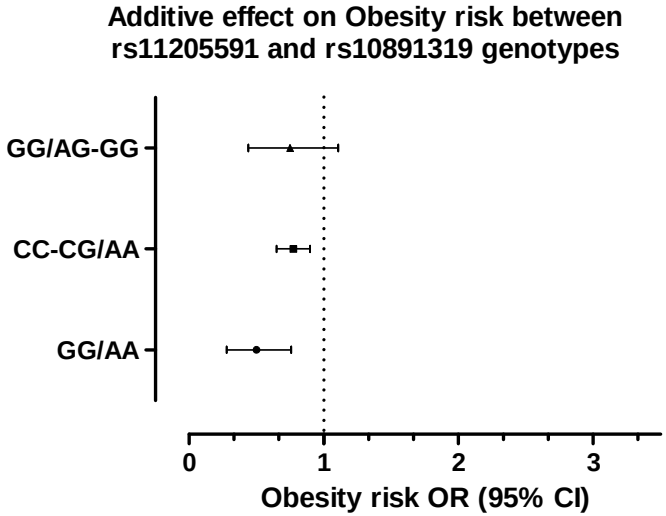

Additive effect on Obesity risk between rs11205591 and rs10891319 genotypes
GG/AG-GG
CC-CG/AA
GG/AA
0
1
2
3
Obesity risk OR (95% CI)

Table 1S: Description of selected SNPs

Gene Name	Chromosome	SNP Name	chromosome position (GRCh38p10)	Most severe consequence	HGVS Name	Reference sequence	MAF	tag SNP
COX5A	15	rs1133322	74920016	DOWNSTREAM	c.*436T>C	NM_004255.3	0.25 (G)	1**
COX5B	2	rs1470625	97649028	DOWNSTREAM	g.97649028C>A	NC_000012.12	0.46 (C)	2**
		rs17022045	97645225	UPSTREAM	c.-862C>G	NM_001862.2	0.13 (G)	1**
COX6A1	12	rs17431357	120442631	UPSTREAM	g.120442631C>T	NC_000012.12	0.04 (T)	
		rs12310837	120442769	UPSTREAM	g.120442769A>G	NC_000012.12	0.15 (G)	
		rs2076022	120437987	UPSTREAM	c.-140C>T	NM_004373.3	0.49 (T)	2**
COX6B1	19	rs4806187	35658859	DOWNSTREAM	c.*212A>G	NM_001863.4	0.33 (A)	1**
COX6C	8	rs1130569	99887565	SYNONYMOUS_CODING	p.Tyr56=	NP_004365.1	0.25 (A)	
		rs4626565	99878314	INTRONIC	c.*16-49A>G	NM_004374.3	0.23 (C)	1**
COX7A1	19	rs753420	36152793	5PRIME_UTR	c.-386A>C	NM_001864.3	0.40 (G)	1**
		rs7255180	36149417	INTRONIC	c.722-161C>T	NM_001003962.2	0.02 (T)	
COX7A2	6	rs436898	75235832	DOWNSTREAM	g.75235832A>C	NC_000006.12	0.16 (A)	
		rs683943	75252092	UPSTREAM	g.75252092C>G	NC_000006.13	0.05 (C)	
		rs9360898	75243989	5PRIME_UTR	c.-159A>C	NM_001865.3	0.21 (G)	3**
COX7B2	4	rs9790574	46732884	DOWNSTREAM	g.46732884C>T	NC_000004.12	0.25 (T)	
COX7C	5	rs16902285	86621753	DOWNSTREAM	g.86621753T>G	NC_000005.10	0.07 (G)	
		rs13161296	86617233	UPSTREAM	c.-823C>T	NM_001867.2	0.01 (T)	1**
		rs2410718	86622655	DOWNSTREAM	g.86622655G>A	NC_000005.10	0.12 (A)	
COX8C	14	rs2089095	93350593	INTRONIC	c.-351+17070C>G	NM_020818.4	0.11 (G)	
NDUFS1	2	rs1053517	206122291	DOWNSTREAM	g.206122291A>G	NC_000002.12	0.49 (G)	1**
		rs3770989	206124027	3PRIME_UTR	c.*158T>C	NM_001199981.1	0.03 (G)	3**
		rs11548670	206147759	SYNONYMOUS_CODING	p.Asp102=	NP_001186910.1	0.07 (G)	2**

Table 1S cont: Description of selected SNPs

Gene Name	Chromosome	SNP Name	chromosome position (GRCh38p10)	Most severe consequence	HGVS Name	Reference sequence	MAF	tag SNP
NDUFS2	1	rs4656994	161210087	INTRONIC	c.703-24G>A	NM_001166159.1	0.26 (A)	3**
		rs1136224	161214307	3PRIME_UTR	c.*366A>G	NM_001166159.1	0.18 (G)	6**
		rs1136207	161213726	SYNONYMOUS_CODING	p.Ala430=	NP_001159631.1	0.16 (T)	
		rs4656993	161206347	INTRONIC	c.203-60A>G	NM_001166159.1	0.30 (A)	
		rs11587213	161215085	UPSTREAM	g.161215085A>G	NC_000001.11	0.15 (G)	5**
NDUFS3	11	rs10742816	47585432	UPSTREAM	g.47585432C>T	NC_000011.10	0.25 (C)	1**
NDUFS4	5	rs31304	53646253	SYNONYMOUS_CODING	p.Gly66=	NP_001304980.1	0.06 (A)	
		rs567	53683267	3PRIME_UTR	c.*46G>A	NM_002495.3	0.38 (A)	3**,5**,6**
		rs535277	53658684	INTRONIC	c.424+60T>G	NM_002495.3	0.29 (G)	
NDUFS5	1	rs1984600	39026255	UPSTREAM	c.-150G>C	NM_004552.2	0.46 (G)	1**
		rs11205591	39035577	DOWNSTREAM	g.39035577C>G	NC_000001.11	0.24 (G)	2**
NDUFS6	5	rs11953620	1818516	DOWNSTREAM	g.1818516C>T	NC_000005.10	0.42 (T)	
		rs4975851	1817222	DOWNSTREAM	g.1817222C>T	NC_000005.10	0.38 (C)	
NDUFS7	19	rs11666067	1389518	non coding transcript exon variant	c.228+580C>A	NM_024407.4	0.46 (A)	
SDHB	1	rs1022580	17033181	INTRONIC	c.201-36G>T	NM_003000.2	0.04 (C)	1**
		rs2647169	17044728	INTRONIC	c.200+33G>A	NM_003000.2	0.11 (T)	3**
		rs11203280	17016288	UPSTREAM	g.17016288G>T	NC_000001.11	0.50 (G)	
		rs9435739	17016429	UPSTREAM	g.17016429G>A	NC_000001.11	0.48 (A)	

Table 1S cont2: Description of selected SNPs

Gene Name	Chromosome	SNP Name	chromosome position (GRCh38p10)	Most severe consequence	HGVS Name	Reference sequence	MAF	tag SNP
SDHC	1	rs4600063	161363401	3PRIME_UTR	g.161363401A>G	NC_000001.11	0.17 (G)	
		rs13374037	161365491	3PRIME_UTR	g.161365491T>A	NC_000001.11	0.17 (A)	3**
SDHD	11	rs10891319	112096881	INTRONIC	g.112096881A>G	NC_000011.10	0.35 (G)	
UQCRB	8	rs10504961	96227901	3PRIME_UTR	g.96227901C>T	NC_000008.11	0.37 (T)	
UQCRQ	5	rs803224	132870812	UPSTREAM	g.132870812G>A	NC_000005.10	0.18 (G)	
		rs17624157	132868339	UPSTREAM	g.132868339G>A	NC_000005.10	0.05 (A)	
GDF9	5	rs39830	132865726	UPSTREAM	g.132865726G>A	NC_000005.10		
		rs30177	132866719	5PRIME_UTR	g.132866719C>G	NC_000005.10	0.36 (C)	3**,4**

SNP Name: dbSNP 149, Ensemble release 89. MAF: Minor allele frequency. HGVS name: nomclature following Human Genome Variation Society guidelines

Table 1. STREGA reporting recommendations, extended from STROBE Statement

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Title and Abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract.		1
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found.		2
Introduction				
Background rationale	2	Explain the scientific background and rationale for the investigation being reported.		3-4
Objectives	3	State specific objectives, including any pre-specified hypotheses.	***State if the study is the first report of a genetic association, a replication effort, or both.***	4
Methods				
Study design	4	Present key elements of study design early in the paper.		5-6

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Setting	5	Describe the setting, locations and relevant dates, including periods of recruitment, exposure, follow-up, and data collection.		5
Participants	6	(a) **Cohort study –** Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up. **Case-control study –** Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls. **Cross-sectional study –** Give the eligibility criteria, and the sources and methods of selection of participants.	***Give information on the criteria and methods for selection of subsets of participants from a larger study, when relevant***.	5
		(b) **Cohort study –** For matched studies, give matching criteria and number of exposed and unexposed. **Case-control study –** For matched studies, give matching criteria and the number of controls per case.		NA
Variables	7	*(a)* Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable.	***(b) Clearly define genetic exposures (genetic variants) using a widely-used nomenclature system. Identify variables likely to be associated with population***	6

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			stratification (confounding by ethnic origin).	
Data sources measurement	8*	*(a)* For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group.	***(b) Describe laboratory methods, including source and storage of DNA, genotyping methods and platforms (including the allele calling algorithm used, and its version), error rates and call rates. State the laboratory/centre where genotyping was done. Describe comparability of laboratory methods if there is more than one group. Specify whether genotypes were assigned using all of the data from the study simultaneously or in smaller batches.***	5-6
Bias	9	*(a)* Describe any efforts to address potential sources of bias.	***(b) For quantitative outcome variables, specify if any investigation of potential bias resulting***	

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			from pharmacotherapy was undertaken. If relevant, describe the nature and magnitude of the potential bias, and explain what approach was used to deal with this.	NA
Study size	10	Explain how the study size was arrived at.		5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why.	***If applicable, describe how effects of treatment were dealt with.***	5-6
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding.	***State software version used and options (or settings) chosen.***	7
		(b) Describe any methods used to examine subgroups and interactions.		7
		(c) Explain how missing data were addressed.		7

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(d) **Cohort study –** If applicable, explain how loss to follow-up was addressed. **Case-control study –** If applicable, explain how matching of cases and controls was addressed. **Cross-sectional study –** If applicable, describe analytical methods taking account of sampling strategy.		NA
		(e) Describe any sensitivity analyses.		
			(f) State whether Hardy-Weinberg equilibrium was considered and, if so, how.	7
			(g) Describe any methods used for inferring genotypes or haplotypes.	7
			(h) Describe any methods used to assess or address population stratification.	NA
			(i) Describe any methods used to address multiple	

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			comparisons or to control risk of false positive findings.	7
			(j) Describe any methods used to address and correct for relatedness among subjects	NA
Results				
Participants	13*	(a) Report the numbers of individuals at each stage of the study – e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed.	***Report numbers of individuals in whom genotyping was attempted and numbers of individuals in whom genotyping was successful.***	5-6
		(b) Give reasons for non-participation at each stage.		5
		(c) Consider use of a flow diagram.		
Descriptive data	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders.	***Consider giving information by genotype.***	5

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Indicate the number of participants with missing data for each variable of interest.		6
		(c) **Cohort study –** Summarize follow-up time, e.g. average and total amount.		NA
Outcome data	15 *	**Cohort study-**Report numbers of outcome events or summary measures over time.	***Report outcomes (phenotypes) for each genotype category over time***	
		Case-control study – Report numbers in each exposure category, or summary measures of exposure.	***Report numbers in each genotype category***	
		Cross-sectional study – Report numbers of outcome events or summary measures.	***Report outcomes (phenotypes) for each genotype category***	10-11
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence intervals). Make clear which confounders were adjusted for and why they were included.		7

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Report category boundaries when continuous variables were categorized.		6
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period.		NA
			(d) Report results of any adjustments for multiple comparisons.	10-11
Other analyses	17	(a) Report other analyses done – e.g., analyses of subgroups and interactions, and sensitivity analyses.		
			(b) If numerous genetic exposures (genetic variants) were examined, summarize results from all analyses undertaken.	10-11
			(c) If detailed results are available elsewhere, state how they can be accessed.	NA
Discussion				
Key results	18	Summarize key results with reference to study objectives.		14

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.		14
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.		14
Generalizability	21	Discuss the generalizability (external validity) of the study results.		14
Other Information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based.		15

STREGA = STrengthening the REporting of Genetic Association studies; STROBE = STtrengthening the Reporting of Observational Studies in Epidemiology.

* Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

BMJ Open

RESPIRATORY CHAIN POLYMORPHISMS AND OBESITY IN THE SPANISH POPULATION, A CROSS-SECTIONAL STUDY

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Secondary Subject Heading:	Diabetes and endocrinology
Keywords:	Obesity, SNP, mitochondrial respiratory chain

RESPIRATORY CHAIN POLYMORPHISMS AND OBESITY IN THE SPANISH POPULATION, A CROSS-SECTIONAL STUDY

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ABSTRACT

OBJECTIVE: To study the association of genes involved in the Mitochondrial Respiratory Chain (MRC) pathway with Body Mass Index (BMI) and obesity risk.

DESIGN: This work studies three cross-sectional populations from Spain, representing three provinces: HORTEGA (Valladolid, Northwest/Center), SEGOVIA (Segovia, Northwest/center), and PIZARRA (Malaga,South).

SETTING: Forty-eight SNPs from MRC genes were selected and genotyped by SNPlex method. Association studies with BMI and obesity risk were performed for each population. These associations were then verified by analysis of the studied population as a whole (3731 samples).

PARTICIPANTS: a total of 3731 Caucasian individuals: 1502 samples from HORTEGA, 988 from PIZARRA and 1241 from SEGOVIA.

RESULTS: rs4600063 (*SDHC*), rs11205591 (*NDUFS5*) and rs10891319 (*SDHD*) SNPs were associated with BMI and obesity risk (p values for BMI were 0.04, 0.0011 and 0.0004, respectively, and for obesity risk, 0.0072, 0.039 and 0.0038). However, associations between rs4600063 and BMI, and between these 3 SNPs and obesity risk are not significant if Bonferroni correction is considered. In addition, rs11205591 and rs10891319 polymorphisms showed an additive interaction with BMI and obesity risk.

CONCLUSIONS: Several polymorphisms from genes coding MRC proteins may be involved in BMI variability and could be related to the risk to become obese in the Spanish general population.

KEYWORDS: Obesity, SNP, mitochondrial respiratory chain,

STRENGHTS AND LIMITATIONS OF THIS STUDY:

- This research was conducted in three open populations from different provinces of Spain.
- MRC SNP analysis by SNPlex genotyping method overcomes some of the limitations of GWAS.
- One of the limitations of this study is the reduced size of the populations used
- Despite the limited number of individuals in this study, the statistical power is sufficient for the number of analyzed SNPs.
- Results from this study are promising and should be validated by larger sample sizes

INTRODUCTION

Obesity is a metabolic disorder consisting of excess body fat accumulation. Obesity prevalence has been increasing during the last decades in Western Societies, becoming one of the most important public health problems due to its causally relationship with several chronic diseases such as insulin resistance (IR), cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM).[1]

Susceptibility to obesity is determined by environmental and genetic factors. Although rising obesity prevalence is triggered by lifestyle changes, the risk of developing obesity has an important genetic component, which has been examined in numerous studies.[2-4] Rare mutations in genes encoding for appetite-regulating proteins, such as leptin (*LEP*) and its receptor (*LEPR*), melanocortin-4 receptor (*MC4R*) and pro-opiomelanocortin (*POMC*), have shown to cause severe early-onset obesity.[5-6] Genome wide association studies (GWAS) have identified common polymorphisms located in, or near to, 97 loci, mostly expressed in the central nervous system.[6-

10] However, it is also suggested that, due to limitations of GWAS, there are around 250 common variants with a similar effect to those previously described that remain to be identified.[8]

The study of target genes or genes involved in a particular physiological pathway is a potential approach to identify genetic variants with roles in complex traits. The mitochondrial respiratory chain (MRC) is a biological system involved in carbohydrate and lipid metabolism through oxidative phosphorylation. MRC produces the proton gradient needed for ATP synthesis and is composed of four large complexes (I to IV) that transport electrons from donors (NADH at complex I, FADH2 at complex II) to acceptors. Finally, electrons in complex IV are conducted to the last acceptor, the molecular oxygen. [11] Mitochondrial oxidative capacity dysfunction in liver, muscle or adipose tissue could contribute to the intracellular accumulation of fatty acids observed in obesity.[12-13] Insulin resistance has been associated with lower mitochondria activity at rest.[14] This may be the result of inherited defects in genes coding MRC proteins.

Mutations of MRC genes resulting in loss of function have been reported to produce major disability-causing diseases.[15-18] In addition, studies on their impact on energy efficiency and expenditure have shown an association with the risk of developing obesity.[12, 19-21] Studies have also shown that obesity affects the function of mitochondria, leading to mitochondrial dysfunction.[22] Mitochondrial dynamics play a role in metabolism and obesity: *MFN2*, a gene coding for a GTPase in the outer mitochondrial membrane is involved in mitochondrial fusion, regulating the operation of the mitochondrial network in skeletal muscle. When *MFN2* is repressed, glucose oxidation and mitochondrial membrane potential are impaired.[23] It has been shown that expression of *MNF2* is repressed in obese patients compared to lean.[23] Furthermore, polymorphisms in MRC genes, or others related to its regulation, have been related to obesity in different populations.[23-25]

The aim of this study was to find associations between MRC nuclear genes, BMI and obesity in three different Spanish studies in the general population.

MATERIALS AND METHODS

Sample Populations

We independently analyzed three general Spanish populations originally recruited for the study of cardiovascular risk factors and cardiovascular disease development: HORTEGA (1502 subjects collected from 2004 to 2005), PIZARRA (988 individuals, collected from 2009 and 2010) and SEGOVIA (1239 subjects, collected between 2000 and 2003). [26-28] The HORTEGA sample involves subjects from the Valladolid area (North-western Spain). The PIZARRA sample comprises subjects from Pizarra, a town in the Malaga province (Andalusia, Southern Spain). The SEGOVIA sample studied subjects from the Segovia Province (Center of Spain). These three populations were recruited to identify cardiovascular risk factors in the general population as an overall goal. Individuals were randomly selected and invited to participate. Exclusion criteria were any concomitant disease or condition that prevented them from answering a survey, or donating a sample or that would influence the collection of reliable information. The study was approved by the Research and Ethics Committee from the Valencia University Clinical Hospital and INCLIVA (reference number 2010/013). All patients provided informed written consent to take part in the investigations. The research was carried out according to the Ethical Principles of the World Medical Association (Declaration of Helsinki)

Demographic data and anthropometric parameters were collected following standard

procedures. Presence of obesity, hypertension (HTN) and Type 2 Diabetes mellitus (T2DM) was recorded. BMI was calculated by dividing weight in kilograms by height in meters squared. Obesity, HTN and T2DM were defined using the World Health Organization (WHO) criteria (http://www.who.int). Briefly, obesity was diagnosed with a BMI >30 kg/m2, overweight as BMI between 25.0 and 29.9 kg/m2, and normal weight as BMI≤24.9 kg/m2. HTN was defined by systolic and diastolic blood pressure above 140 or 90 mm Hg, respectively. T2DM was defined by fasting plasma glucose ≥ 126mg/dl or 2-hour plasma glucose ≥ 200mg/dl. Previous diagnosis of T2DM or HTN and detection of the disease at the moment of sample collection were recorded. Missing data for parameters regarding this work (expressed as HORTEGA missing data / PIZARRA missing data / SEGOVIA missing data) were 58/58/6 for BMI and 1/85/63 for glucose.

We have calculated the statistical power for our three samples independently (minor allele frequency (MAF) >0.10, genotype relative risk (1.5)), number of obese and non-obese and prevalence of obesity in each of the populations. Furthermore, the statistical power was over 85% for this conditions in all populations and it increases for increased allele frequency (http://csg.sph.umich.edu/abecasis/cats/gas_power_calculator/index.html)·

Genotyping methods

Gene and SNP selection:

Forty-eight single nucleotide polymorphisms (SNPs) of chromosomal genes coding for MRC proteins were selected for genotyping. Selection was performed based on the following considerations: functionality (previously described or possible effect), MAF ≥1%, representation of genetic variability of the whole gene (HapMap polymorphisms), and spacing along the gene. Most important variants described in the literature were included. Details of genes and SNPs

included are shown as supplemental material (Table 1S).

Genotyping procedure:

Venous blood samples were collected in tubes containing EDTA. DNA was isolated by standard commercial procedures (Chemagic Magnetic Separator from Chemagen, Baesweiler, Germany). DNA was quantified and diluted to a final concentration of 100 ng/ul.

SNPlex (Applied Biosystems, Foster City, California, USA) was used for genotyping, following the manufacturer's guidelines. SNPlex is a genotyping system based on oligonucleotide ligation assay/polymerase chain reaction technology that analyzes 48 SNPs. Those SNPs were chosen as explained above.

Statistical analysis

Statistical analyses were performed using SPSS version 19 and SNPStats software. [29]

Chi-squared test and variance analysis were used to compare quantitative and categorical variables between groups in order to assess general characteristics of the studied populations. P-values ≤ 0.05 were considered significant.

When analyzing for associations between SNPs and obesity traits in the three populations, 11 out of the 48 SNPs were excluded from the study because they did not fulfill the Hardy-Weimberg equilibrium, had low frequency, or were not detectable by the genotyping procedure. Hardy-Weimberg test indicated no loss of heterozigosity in the analyzed populations for these SNPs. The Bonferroni correction cut off to assess significant associations was calculated for the 37 remaining SNPs. Those polymorphisms associated in at least one of the three populations and showing the same tendency in the remaining ones, or results with p-values near the nominal cut off point in the three studies ($p<0.05$), were also analyzed in the 3729 total combined subjects from

the three populations. For these analyses, multivariate logistic regression under co-dominant model was performed and, if applicable, dominant, recessive, or additive models were used. For categorical variables, adjusted odds ratios were assessed with 95% confidence interval. The association between polymorphisms and BMI values was examined using analysis of covariance (ANCOVA). All p-values were two-sided. All results were obtained after adjustment for age and gender, and p-values <0.05 were considered significant.

Patient and Public involvement

Patients and public were not actively involved in this research. They were informed regarding the research goals, protocol development and parameters to be measured before starting the study. If appropriate, they were informed regarding results.

RESULTS

Characteristics of the studied populations

General characteristics of the studied populations are summarized in Table 1. Age, gender and BMI varied between the three populations. There were differences in obesity and T2DM percentage: obesity prevalence was 17%, 26% and 33% for Hortega, Segovia and Pizarra studies, respectively, while T2DM prevalence was 7.6%, 10.2% and 19.6%. Increased means of age correspond to the age structure of our population. BMI means correspond to overweight values, what is in agreement with the fact that obesity is increasing in Western Societies (WHO).

Table 1: General features of Hortega, Pizarra and Segovia patients considered individually and as a whole population

	Hortega	Pizarra	Segovia	Whole Population
N	1502	988	1241	3731
Age (Years)	54.4 ± 19.3*	46.1 ± 13.9***	51.9 ± 10.8**	51.4 ± 15.9
Height (m)	163.7 ± 10.0*	161 ± 8.8	161.4 ± 9.0**	162.2 ± 9.4
Weight (Kg)	70.8 ± 12.9*	74.1 ± 4.3***	72.1 ± 12.4**	72.1 ± 13.2
BMI (Kg/m2)	26.4 ± 4.2*	28.6 ± 5.3***	27.7 ± 4.3**	27.4 ± 4.6
Gender M(%)/F(%)	754(50.2) / 748(49.8)*	365(36.9) / (616(62.3)***	562(45.3) / (679(54.7)**	2043(54.8) / (1681(45.1)
Obesity (N(%))	262(17.4)*	331(33.5)***	319(25.7)**	912(24.4)

M: Male. F: Female. Values expressed as mean ± SD except for gender and obesity. p value <0.05was considered significant. *for p<0.05 when comparing Hortega with Pizarra, ** p<0.05 when comparing Hortega and Segovia, *** for p<0.05 when comparing Pizarra and Segovia

Association between MRC genes SNPs and obesity

We performed the analysis looking for associations between SNPs, BMI and obesity risk in the three independent populations. Table 2 shows results for SNPS with differences in at least one of the three populations and a similar trend in the remaining ones, or that had p-values near the nominal cut-off point (p≤0.05) in the three studies: rs1136224 (*NDUFS2* gene), rs11205591

(*NDUFS5*), rs4600063 (*SDHC*), rs3770989 (*NDUFS1*) and rs10891319 (*SDHD*). These five SNPs were selected for further analysis in the whole population.

Table 2: BMI and obesity risk of associated SNPs for Hortega, Pizarra and Segovia populations.

		HORTEGA			PIZARRA			SEGOVIA		
	Geno-type	**N**	**BMI (Kg/m2)**	**Obesity OR (95%CI)**	**N**	**BMI (Kg/m2)**	**Obesity OR (95%CI)**	**N**	**BMI (Kg/m2)**	**Obesity OR (95%CI)**
SDHC	AA	1250	26.46±0.12	1	772	28.70±0.19	1	943	27.71±0.10	1
rs4600063	AG-GG	183	26.16±0.30	0.82 (0.55-1.22)	96	27.86±.04	0.47 (0.28-0.78)	132	27.21±0.30	0.88 (0.57-1.36)
HWE:1	p-value		0.9	0.33		0.071	**0.0025**		0.22	0.57
NDUFS1	TT	1308	26.41±0.12	1	799	28.49±0.10	1	1028	27.56±0.10	1
rs3770989	CT-CC	114	26.62±0.44	1.13 (0.72-1.77)	77	29.85±0.60	1.5 (0.91-2.48)	65	27.92±0.60	1.28 (0.73-2.23)
HWE:1	p-value		0.34	0.6		**0.016**	0.12		0.48	0.39
NDUFS2	AA	1045	26.37±0.13	1	640	28.61±0.21	1	830	27.55±0.15	1
rs1136224	AG-GG	369	26.59±0.23	1.43 (1.09-1.89)	223	28.59±0.34	1.10 (0.79-1.54)	241	27.77±0.26	1.07 (0.76-1.48)
HWE:0.36	p-value		0.25	**0.011**		0.71	0.58		0.42	0.71
NDUFS5	CC-CG	1309	26.47±0.12	1	832	28.63±0.10	1	947	27.6±0.14	1
rs11205591	GG	115	25.78±0.31	0.67 (0.41-1.10)	38	27.66±0.70	0.86 (0.41-1.81)	89	27.21±0.40	0.89 (0.53-1.5)
HWE:0.76	p-value		**0.049**	0.11		0.38	0.69		0.25	0.66
SDHD	AA	691	26.16±0.16	1	401	28.18±0.26	1	571	27.37±0.17	1
rs10891319	AG-GG	726	26.64±0.16	1.16 (0.90-1.49)	478	29.00±0.24	1.22 (0.91-1.64)	492	27.83±0.19	1.27 (0.96-1.68)
HWE:0.22	p-value		0.08	0.25		0.084	0.18		0.077	0.095

BMI: mean ± SD. OR: odds ratio; 95%CI: 95% confidence interval. HWE: Hardy Weinberg Equilibrium. N: Number of individuals. Bold: significant

Analyzing these five SNPS in the total population, four polymorphisms were associated with BMI: rs4600063, rs10891319, rs3770989, rs11205591, located in the *SDHC, SDHD, NDUFS1* and *NDUFS5,* genes respectively. Results for these four SNPs are shown in Table 3: rs4600063 and rs3770989 showed low association with BMI, which lost significance after

Bonferroni correction (p<0.0013). Rs10891319 and rs11205591 showed significant association with BMI after Bonferroni correction, with a p-value of 0.0004 for the first one and 0.0011 for the second one. On the other hand, rs4600063 (genotypes AG and GG) and rs11205591 (GG genotype) reduced obesity risk, while rs10891319 (AG and GG genotypes) and rs1136224 (AG and GG genotypes) increased the risk (p<0.05), but they did not reach the Bonferroni cut-off point.

Table 3: SNP Genotyping: distribution of BMI levels and the associated obesity risk for all genotypes in whole population.

Gene/SNP	Genotype	N	BMI (Kg/m²)	Non Obesity (N(%))	Obesity (N(%))	OR (95% CI)
SDHC	AA	2965	27.44±0.08	2109(86.9)	851(90.4)	1
rs4600063	AG-GG	411	26.89±0.21	318(13.1)	90(9.6)	0.72 (0.56-0.92)
	p-value		**0.04**			**0.0072**
SDHD	AA	1663	27.06±0.11	1243(51.3)	422(45.4)	1
rs10891319	AG-GG	1696	27.60±0.11	1178(48.7)	508(54.6)	1.25 (1.08-1.46)
	p-value		**0.0004**			**0.0038**
NDUFS1	TT	3135	27.32±0.08	2273(92.9)	857(91.2)	1
rs3770989	CT-CC	256	27.92±0.32	173(7.1)	83(8.8)	1.30 (0.99-1.72)
	p-value		**0.026**			0.066
NDUFS2	AA	2515	27.33±0.09	1833(76.1)	680(73)	1
rs1136224	AG-GG	833	27.46±0.16	575(23.9)	251(27)	1.20(1.01-1.43)
	p-value		0.31			**0.041**
NDUFS5	CC-CG	**3088**	27.40±0.08	2219(92.3)	863(94)	1
rs11205591*	GG	**242**	26.60±0.24	184(7.7)	55(6)	0.72 (0.52-0.99)
	p-value		**0.0011**			**0.039**

OR: odds ratio; 95%CI: 95% confidence interval. BMI values are expressed as mean ± standard deviation. Results after adjustment for the following variables included in the model: age and gender. N: Number of individuals. *tag-SNP.

Figure 1 shows BMI differences for rs10891319 and rs11205591 polymorphisms in each independent population and as a whole. A similar trend can be observed in both figures 1A and 1B, although only the rs11205591 SNP reaches significance in the HORTEGA study ($p \leq 0.05$).

We studied additive effects for all these SNPs and an additive effect for rs11205591 and rs10891319 SNPs was found in the whole sample: patients carrying GG/AA combination presented significant lower BMI (Figure 2A) and lower obesity risk (Figure 2B) than patients carrying CC-CG / AG-GG genotypes (data are expressed as rs11205591 genotype / rs10891319 genotype).

On the other hand, some of the SNPs were within the gene. We calculated possible haplotypes, with negative results.

DISCUSSION

The goal of this population-based study was to analyze MRC SNPs and their association with BMI and obesity risk in three Spanish populations. SNPs with the most relevant associations were tested in the pooled sample.

We found a significant protective association with the risk of obesity for rs10891319 AA, and for patients with the rs11205591 GG genotype, who also showed reduced BMI. Despite differences in age, sex, BMI, and obesity prevalence between the three samples, a similar trend was found considering them individually, while rs10891319 showed the strongest in the whole

sample. Furthermore, an additive interaction was observed between these two SNPs, with a maximal BMI difference of 1.4 kg/m2 found between genotypes (CC-GG / AA-GG vs GG / AA carriers, expressed as rs11205591/ rs10891319 genotype carriers). In agreement with these findings, significant differences in obesity risk were also found.

Another SNP associated with BMI in one of the three populations and in the whole group, rs3770989, is located in the 3'UTR region of the *NDUFS1* gene, which codes for NADH dehydrogenase (ubiquinone) Fe-S protein 1. In silico analysis in TargetScanHuman (http://www.targetscan.org/vert_50/) showed that this SNP is located between two regions of miRNA binding sites, although it does not seem to affect either of them.[30,31] On the other hand, the rs11205591 and rs10891319 SNPs are located in 3' regions of *NDUFS5* and *SDHD* genes respectively. Although these SNPs have an unknown functional effect, they could be in linkage disequilibrium with other truly functional polymorphisms. New studies are needed to address this question.

The first gene consistently identified as an obesity gene using GWAS was FTO. Several SNPs located on its first intron were associated with BMI in European, East Asian and African populations. [32,33] Following this gene, 75 additional obesity loci have been identified using this methodology and, although FTO is the gene accounting for most of the inter-individual variability on BMI, it only explains a 0.34%.[8,33] There are data supporting that alterations in genes related to mitochondrial function can be involved in obesity, [12,15,20,21,34] but few studies have analyzed the effect of its variations on BMI or obesity.[19,24] From all genes identified in the GWAS meta-analysis performed by Speliotes and colleagues,[8] only the *NDUFS3* (NADH dehydrogenase (ubiquinone) Fe-S protein 3) gene was identified to be within 300 Kb from the BMI associated SNPs. As these authors indicated, in spite of the large population analyzed in their study, many genetic variants related to BMI and obesity remain to be identified. The limitations

of this meta-analysis come from the different microarrays used, with low coverage in many genomic regions. Therefore, the *NDUFS5* gene (where rs11205591 is located) has been analyzed by only one polymorphism in each of the two most used microarrays by Speliotes and in most of the GWAS performed to date;[8] (http://www.Illumina.com; http://www.affymetrix.com; http://www.ensembl.org). In addition, representation of Mediterranean populations in Speliotes and Locke´s studies is very small, and, to our knowledge, Spanish populations are not included.[8,9] Therefore, many important genetic regions, related to BMI and obesity regulation in these populations may be missing from their work.

The present study supports the hypothesis that genetic variations in MRC genes may be related to obesity susceptibility in the Spanish population. Therefore, these genes could be new therapeutic targets. Genetics of obesity are complex and not yet well identified, and our data may contribute to a better understanding. One limitation of this study is the reduced size of the analyzed populations. However, the statistical power is sufficient for the number of analyzed SNPs. On the other hand, we have not studied all the possible SNPs present in these MRC genes. Further functional studies and association analyses in larger samples and other populations should be carried out to confirm our results.

AUTHOR CONTRIBUTIONS

G.DM-S. obtained genetic data, analyzed and interpreted results and wrote the manuscript**. VGA** obtained genetic data, contributed to analysis and interpretation and reviewed the manuscript. **JTR, LSBF, MCS, ALS, and AC** obtained population data, made critical revisions of the manuscript and contributed to the discussion. **JCME, GRM, RC**, and **MTML** contributed to population studies and discussion, and reviewed and edited the manuscript. **FJC** and **ABGG**

designed the study, wrote the manuscript, contributed to discussion and reviewed and edited the manuscript.

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DISCLAIMER

Neither funder played a role in the study design, data analysis or interpretation of the data.

COMPETING INTERESTS

There are no competing interests to declare

DATA SHARING STATEMENT

No additional data are available.

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LEGENDS

Figure 1. BMI differences found for the associated SNPs genotypes for each population analyzed in this study. Values are expressed as BMI difference means, showing 95% confidence interval. **A**) rs10891319 BMI differences between AG-GG and AA genotypes, taking the first one (BMI difference=0 for carriers of AG-GG genotype) as reference. **B)** rs11205591 BMI differences for CC-CG and GG genotypes, with the first one as a reference (CC-CG genotype BMI difference=0).

Figure 2. Additive effect of rs10891319 and rs11205591 genotype combination in BMI and obesity risk. **A)** BMI means. * indicates a p-value < 0.005. **B)** Obesity risk ORs . Rs11205591 CC-CG / rs10891319 AA genotypes were taken as a reference (OR=1). The obtained p-value was <0.003. Error bars show standard error. Data are expressed as rs11205591 / rs10891319 genotypes

Figure 1

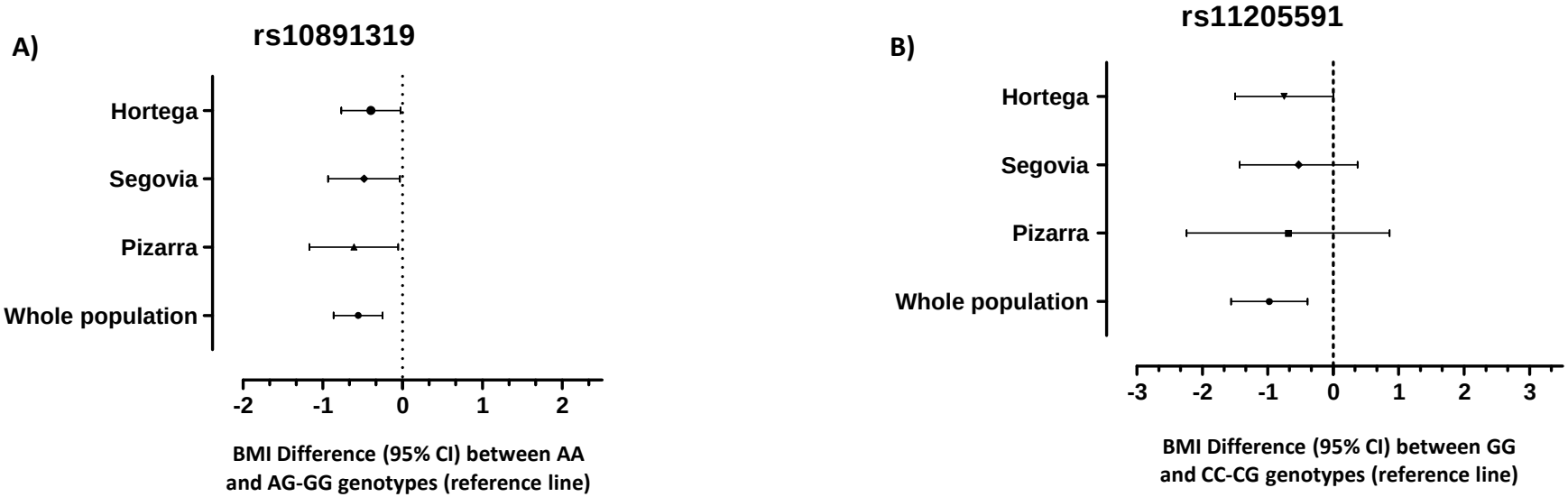
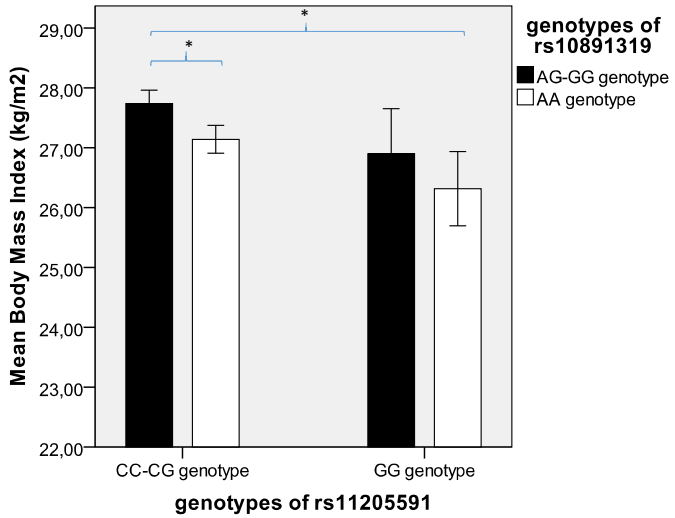

A)
rs10891319
Hortega
Segovia
Pizarra
Whole population
-2
-1
0
1
2
BMI Difference (95% CI) between AA and AG-GG genotypes (reference line)
B)
rs11205591
Hortega
Segovia
Pizarra
Whole population
-3
-2
-1
0
1
2
3
BMI Difference (95% CI) between GG and CC-CG genotypes (reference line)

Figure 2.

A)


Mean Body Mass Index (kg/m2)
29,00
28,00
27,00
26,00
25,00
24,00
23,00
22,00
*
*
genotypes of rs10891319
AG-GG genotype
AA genotype
CC-CG genotype
GG genotype
genotypes of rs11205591

B)

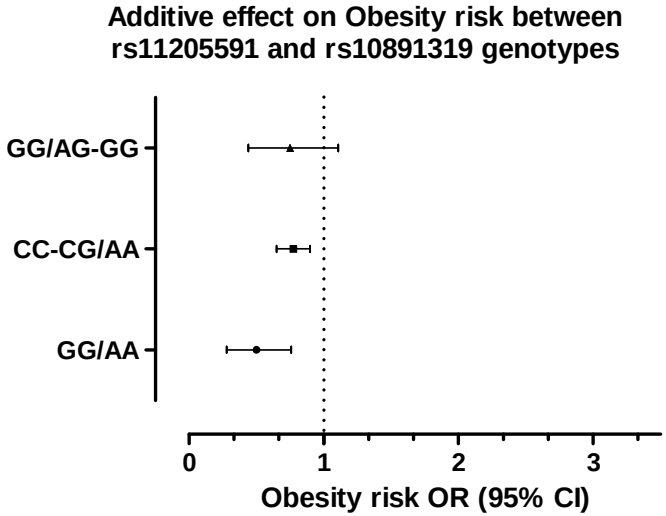

Additive effect on Obesity risk between rs11205591 and rs10891319 genotypes
GG/AG-GG
CC-CG/AA
GG/AA
0
1
2
3
Obesity risk OR (95% CI)

Table 1S: Description of selected SNPs

Gene Name	Chromosome	SNP Name	chromosome position (GRCh38p10)	Most severe consequence	HGVS Name	Reference sequence	MAF	tag SNP
COX5A	15	rs1133322	74920016	DOWNSTREAM	c.*436T>C	NM_004255.3	0.25 (G)	1**
COX5B	2	rs1470625	97649028	DOWNSTREAM	g.97649028C>A	NC_000012.12	0.46 (C)	2**
		rs17022045	97645225	UPSTREAM	c.-862C>G	NM_001862.2	0.13 (G)	1**
COX6A1	12	rs17431357	120442631	UPSTREAM	g.120442631C>T	NC_000012.12	0.04 (T)	
		rs12310837	120442769	UPSTREAM	g.120442769A>G	NC_000012.12	0.15 (G)	
		rs2076022	120437987	UPSTREAM	c.-140C>T	NM_004373.3	0.49 (T)	2**
COX6B1	19	rs4806187	35658859	DOWNSTREAM	c.*212A>G	NM_001863.4	0.33 (A)	1**
COX6C	8	rs1130569	99887565	SYNONYMOUS_CODING	p.Tyr56=	NP_004365.1	0.25 (A)	
		rs4626565	99878314	INTRONIC	c.*16-49A>G	NM_004374.3	0.23 (C)	1**
COX7A1	19	rs753420	36152793	5PRIME_UTR	c.-386A>C	NM_001864.3	0.40 (G)	1**
		rs7255180	36149417	INTRONIC	c.722-161C>T	NM_001003962.2	0.02 (T)	
COX7A2	6	rs436898	75235832	DOWNSTREAM	g.75235832A>C	NC_000006.12	0.16 (A)	
		rs683943	75252092	UPSTREAM	g.75252092C>G	NC_000006.13	0.05 (C)	
		rs9360898	75243989	5PRIME_UTR	c.-159A>C	NM_001865.3	0.21 (G)	3**
COX7B2	4	rs9790574	46732884	DOWNSTREAM	g.46732884C>T	NC_000004.12	0.25 (T)	
COX7C	5	rs16902285	86621753	DOWNSTREAM	g.86621753T>G	NC_000005.10	0.07 (G)	
		rs13161296	86617233	UPSTREAM	c.-823C>T	NM_001867.2	0.01 (T)	1**
		rs2410718	86622655	DOWNSTREAM	g.86622655G>A	NC_000005.10	0.12 (A)	
COX8C	14	rs2089095	93350593	INTRONIC	c.-351+17070C>G	NM_020818.4	0.11 (G)	
NDUFS1	2	rs1053517	206122291	DOWNSTREAM	g.206122291A>G	NC_000002.12	0.49 (G)	1**
		rs3770989	206124027	3PRIME_UTR	c.*158T>C	NM_001199981.1	0.03 (G)	3**
		rs11548670	206147759	SYNONYMOUS_CODING	p.Asp102=	NP_001186910.1	0.07 (G)	2**

Table 1S cont: Description of selected SNPs

Gene Name	Chromosome	SNP Name	chromosome position (GRCh38p10)	Most severe consequence	HGVS Name	Reference sequence	MAF	tag SNP
NDUFS2	1	rs4656994	161210087	INTRONIC	c.703-24G>A	NM_001166159.1	0.26 (A)	3**
		rs1136224	161214307	3PRIME_UTR	c.*366A>G	NM_001166159.1	0.18 (G)	6**
		rs1136207	161213726	SYNONYMOUS_CODING	p.Ala430=	NP_001159631.1	0.16 (T)	
		rs4656993	161206347	INTRONIC	c.203-60A>G	NM_001166159.1	0.30 (A)	
		rs11587213	161215085	UPSTREAM	g.161215085A>G	NC_000001.11	0.15 (G)	5**
NDUFS3	11	rs10742816	47585432	UPSTREAM	g.47585432C>T	NC_000011.10	0.25 (C)	1**
NDUFS4	5	rs31304	53646253	SYNONYMOUS_CODING	p.Gly66=	NP_001304980.1	0.06 (A)	
		rs567	53683267	3PRIME_UTR	c.*46G>A	NM_002495.3	0.38 (A)	3**,5**,6**
		rs535277	53658684	INTRONIC	c.424+60T>G	NM_002495.3	0.29 (G)	
NDUFS5	1	rs1984600	39026255	UPSTREAM	c.-150G>C	NM_004552.2	0.46 (G)	1**
		rs11205591	39035577	DOWNSTREAM	g.39035577C>G	NC_000001.11	0.24 (G)	2**
NDUFS6	5	rs11953620	1818516	DOWNSTREAM	g.1818516C>T	NC_000005.10	0.42 (T)	
		rs4975851	1817222	DOWNSTREAM	g.1817222C>T	NC_000005.10	0.38 (C)	
NDUFS7	19	rs11666067	1389518	non coding transcript exon variant	c.228+580C>A	NM_024407.4	0.46 (A)	
SDHB	1	rs1022580	17033181	INTRONIC	c.201-36G>T	NM_003000.2	0.04 (C)	1**
		rs2647169	17044728	INTRONIC	c.200+33G>A	NM_003000.2	0.11 (T)	3**
		rs11203280	17016288	UPSTREAM	g.17016288G>T	NC_000001.11	0.50 (G)	
		rs9435739	17016429	UPSTREAM	g.17016429G>A	NC_000001.11	0.48 (A)	

Table 1S cont2: Description of selected SNPs

Gene Name	Chromosome	SNP Name	chromosome position (GRCh38p10)	Most severe consequence	HGVS Name	Reference sequence	MAF	tag SNP
SDHC	1	rs4600063	161363401	3PRIME_UTR	g.161363401A>G	NC_000001.11	0.17 (G)	
		rs13374037	161365491	3PRIME_UTR	g.161365491T>A	NC_000001.11	0.17 (A)	3**
SDHD	11	rs10891319	112096881	INTRONIC	g.112096881A>G	NC_000011.10	0.35 (G)	
UQCRB	8	rs10504961	96227901	3PRIME_UTR	g.96227901C>T	NC_000008.11	0.37 (T)	
UQCRQ	5	rs803224	132870812	UPSTREAM	g.132870812G>A	NC_000005.10	0.18 (G)	
		rs17624157	132868339	UPSTREAM	g.132868339G>A	NC_000005.10	0.05 (A)	
GDF9	5	rs39830	132865726	UPSTREAM	g.132865726G>A	NC_000005.10		
		rs30177	132866719	5PRIME_UTR	g.132866719C>G	NC_000005.10	0.36 (C)	3**,4**

SNP Name: dbSNP 149, Ensemble release 89. MAF: Minor allele frequency. HGVS name: nomclature following Human Genome Variation Society guidelines

Table 1. STREGA reporting recommendations, extended from STROBE Statement

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Title and Abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract.		1
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found.		2
Introduction				
Background rationale	2	Explain the scientific background and rationale for the investigation being reported.		3-4
Objectives	3	State specific objectives, including any pre-specified hypotheses.	***State if the study is the first report of a genetic association, a replication effort, or both.***	4
Methods				
Study design	4	Present key elements of study design early in the paper.		5-6

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Setting	5	Describe the setting, locations and relevant dates, including periods of recruitment, exposure, follow-up, and data collection.		5
Participants	6	(a) **Cohort study –** Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up. **Case-control study –** Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls. **Cross-sectional study –** Give the eligibility criteria, and the sources and methods of selection of participants.	***Give information on the criteria and methods for selection of subsets of participants from a larger study, when relevant***.	5
		(b) **Cohort study –** For matched studies, give matching criteria and number of exposed and unexposed. **Case-control study –** For matched studies, give matching criteria and the number of controls per case.		NA
Variables	7	*(a)* Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable.	***(b) Clearly define genetic exposures (genetic variants) using a widely-used nomenclature system. Identify variables likely to be associated with population***	6

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			stratification (confounding by ethnic origin).	
Data sources measurement	8*	*(a)* For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group.	***(b) Describe laboratory methods, including source and storage of DNA, genotyping methods and platforms (including the allele calling algorithm used, and its version), error rates and call rates. State the laboratory/centre where genotyping was done. Describe comparability of laboratory methods if there is more than one group. Specify whether genotypes were assigned using all of the data from the study simultaneously or in smaller batches.***	5-6
Bias	9	*(a)* Describe any efforts to address potential sources of bias.	***(b) For quantitative outcome variables, specify if any investigation of potential bias resulting***	

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			from pharmacotherapy was undertaken. If relevant, describe the nature and magnitude of the potential bias, and explain what approach was used to deal with this.	NA
Study size	10	Explain how the study size was arrived at.		5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why.	***If applicable, describe how effects of treatment were dealt with.***	5-6
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding.	***State software version used and options (or settings) chosen.***	7
		(b) Describe any methods used to examine subgroups and interactions.		7
		(c) Explain how missing data were addressed.		7

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(d) **Cohort study –** If applicable, explain how loss to follow-up was addressed. **Case-control study –** If applicable, explain how matching of cases and controls was addressed. **Cross-sectional study –** If applicable, describe analytical methods taking account of sampling strategy.		NA
		(e) Describe any sensitivity analyses.		
			(f) State whether Hardy-Weinberg equilibrium was considered and, if so, how.	7
			(g) Describe any methods used for inferring genotypes or haplotypes.	7
			(h) Describe any methods used to assess or address population stratification.	NA
			(i) Describe any methods used to address multiple	

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			comparisons or to control risk of false positive findings.	7
			(j) Describe any methods used to address and correct for relatedness among subjects	NA
Results				
Participants	13*	(a) Report the numbers of individuals at each stage of the study – e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed.	***Report numbers of individuals in whom genotyping was attempted and numbers of individuals in whom genotyping was successful.***	5-6
		(b) Give reasons for non-participation at each stage.		5
		(c) Consider use of a flow diagram.		
Descriptive data	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders.	***Consider giving information by genotype.***	5

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Indicate the number of participants with missing data for each variable of interest.		6
		(c) **Cohort study –** Summarize follow-up time, e.g. average and total amount.		NA
Outcome data	15 *	**Cohort study-**Report numbers of outcome events or summary measures over time.	***Report outcomes (phenotypes) for each genotype category over time***	
		Case-control study – Report numbers in each exposure category, or summary measures of exposure.	***Report numbers in each genotype category***	
		Cross-sectional study – Report numbers of outcome events or summary measures.	***Report outcomes (phenotypes) for each genotype category***	10-11
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence intervals). Make clear which confounders were adjusted for and why they were included.		7

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Report category boundaries when continuous variables were categorized.		6
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period.		NA
			(d) Report results of any adjustments for multiple comparisons.	10-11
Other analyses	17	(a) Report other analyses done – e.g., analyses of subgroups and interactions, and sensitivity analyses.		
			(b) If numerous genetic exposures (genetic variants) were examined, summarize results from all analyses undertaken.	10-11
			(c) If detailed results are available elsewhere, state how they can be accessed.	NA
Discussion				
Key results	18	Summarize key results with reference to study objectives.		14

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.		14
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.		14
Generalizability	21	Discuss the generalizability (external validity) of the study results.		14
Other Information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based.		15

STREGA = STrengthening the REporting of Genetic Association studies; STROBE = STtrengthening the Reporting of Observational Studies in Epidemiology.

* Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.